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CHROMOSOMAL DNA VARIATION IN *BORDETELLA* SPECIES.

Antonia F. Leigh

**Presented for the degree of Doctor of Philosophy
in the Faculty of Science, University of Glasgow.**

Department of Microbiology

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DEDICATION

I would like to dedicate this thesis to my family and friends for their support and in particular to my mother for her continued inspiration.

DECLARATION

This is the original work of the author
Antonia F. Leigh.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Drs John Coote and Roger Parton, Department of Microbiology, Glasgow University, and Dr Clive Duggleby, Division of Biotechnology, PHLS CAMR, Porton Down, for their constant support throughout this study. I would also like to thank Professors Freer and Wardlaw, Glasgow University, and Professor Atkinson, PHLS CAMR, for providing facilities to work in their departments. Special thanks are due to Roy Hartwell and Kenny Fantom for the synthesis of oligonucleotides. In addition I would like to thank all those I have met whilst at Glasgow University and PHLS CAMR for their advice and encouragement.

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SUMMARY OF RESEARCH

Previous studies had shown that a probe, containing a *hin* DNA invertase gene from *S. typhimurium*, hybridised to a *B. pertussis* chromosomal DNA fragment. This *B. pertussis* fragment was sequenced in this study. Analysis of the DNA and deduced protein sequence showed no homology to DNA invertases nor to other sequences except limited homology to insertion sequence transposases. Reanalysis of the hybridisation of the *hin* DNA fragment probe to chromosomal *Bordetella* DNA showed greater homology to a different fragment. It was deduced that this hybridisation may have been due to flagellin sequences present in the probe. A PCR-amplified *hin* probe derived from *S. typhimurium* DNA was therefore produced consisting only of *hin* DNA. This probe hybridised to tandem repeat sequences in *B. pertussis* DNA. These are known to be insertion sequences. It is hypothesised that the hybridisations to the DNA of the other *Bordetella* strains in this study were also due to limited homology to insertion sequences.

A PCR-amplified *S. typhimurium* H1 flagellin gene, and an oligonucleotide consisting of conserved N-terminal flagellin sequences, were also used as probes. They hybridised to the DNA of all four *Bordetella* strains although only two are recognised as expressing flagella. The two non-motile human pathogens *B. pertussis* and *B. paraptussis* thus both probably contain flagellin genes that are not expressed.

The methylation state of various phases and modes of the *Bordetella* DNA was examined. Previous studies had suggested that *B. pertussis* strains in the virulent phase were modified, possibly by methylation. Results in this study showed the presence of Dcm methylation in the DNA of some of the phase IV

avirulent strains of *B. pertussis* and also under modulated conditions in a virulent phase I strain. This might suggest that other additional modes of phase variation and regulation of virulence factors exist in addition to those already recognised.

ABBREVIATIONS.

AC	Adenylate cyclase enzyme.
ACT	Adenylate cyclase toxin.
ADP	Adenosine diphosphate.
AGG	Agglutininogen.
ATP	Adenosine triphosphate.
bp	Base pairs.
BGA	Bordet-Gengou agar.
Bvg (<i>bvg</i>)	<i>Bordetella</i> virulence protein (gene).
CAA	Casamino acids.
cAMP	Cyclic-adenosine monophosphate.
CAMR	Centre for Applied Microbiology and Research.
Cin (<i>cin</i>)	P1 DNA invertase protein (gene).
cix	<i>cin</i> crossover.
CsCl	Caesium chloride.
<i>cyaA</i>	Adenylate cyclase or cyclolysin gene.
Dam (<i>dam</i>)	DNA adenine methylase protein (gene).
Dcm (<i>dcm</i>)	DNA cytosine methylase protein (gene).
dATP	Deoxy-adenosine triphosphate.
dCTP	Deoxy-cytosine triphosphate.
dGTP	Deoxy-guanosine triphosphate.
dTTP	Deoxy-thymidine triphosphate.
Din	DNA invertase protein.
DNA	Deoxyribonucleic acid.
DTP	Diphtheria, tetanus and pertussis vaccine.

DTT	Dithiothreitol.
DW	Distilled water.
EDTA	Ethylenediamine tetra-acetic acid.
FHA (<i>fha</i>)	Filamentous haemagglutinin (gene).
<i>fim</i>	Fimbrial gene.
FIS	Factor for inversion stimulation.
<i>flaA</i>	Flagellin gene.
<i>fliC</i>	<i>S. typhimurium</i> phase 1, <i>H1</i> flagellin gene, new nomenclature.
<i>fljA</i>	Repressor gene of phase 1 flagellin gene of <i>S. typhimurium</i> , new nomenclature.
<i>fljB</i>	<i>S. typhimurium</i> phase 2, <i>H2</i> flagellin gene, new nomenclature.
g	Gramme.
G _i	Guanine nucleotide binding protein with inhibiting activity.
Gin (<i>gin</i>)	Mu DNA invertase (gene).
<i>gix</i>	<i>gin</i> crossover.
H1 (<i>H1</i>)	<i>S. typhimurium</i> phase 1 flagellin structural protein (gene).
H2 (<i>H2</i>)	<i>S. typhimurium</i> phase 2 flagellin structural protein (gene).
HA	Haemagglutination.
<i>hag</i>	<i>E. coli</i> flagellin gene.
Hin (<i>hin</i>)	<i>S. typhimurium</i> DNA invertase (gene).
<i>hix</i>	<i>hin</i> crossover.
HLT (<i>hlt</i>)	Heat-labile toxin (gene).
HLY	Haemolysin.
HSF	Histamine-sensitising factor.
IAP	Islets-activating protein.
IPTG	Isopropyl beta-D-thiogalactopyranoside.

IS	Insertion sequence.
kb	Kilobase pair.
kD	Kilo Dalton.
LOS	Lipooligosaccharide.
LPF	Lymphocytosis-promoting factor.
LPS	Lipopolysaccharide.
M	Molar.
MAbs	Monoclonal antibodies.
mg	Milligram.
ml	Millilitre.
mM	Millimolar.
<i>mod</i>	Environmental sensor/regulator gene.
Mr	Molecular size.
NA	Nutrient agar.
NB	Nutrient broth.
OMP	Outer-membrane protein.
ORF	Open reading frame.
P	Promoter.
PBS	Phosphate-buffered saline.
PHLS	Public Health Laboratory Service.
Pin (<i>pin</i>)	<i>E. coli</i> DNA invertase (gene).
<i>pix</i>	<i>pin</i> crossover.
PT (<i>ptx</i>)	Pertussis toxin (gene).
PTd	Toxoided pertussis toxin.
P. 69	<i>B. pertussis</i> 69kD outer membrane protein.
P. 68	<i>B. bronchiseptica</i> 68kD outer-membrane protein.
P. 70	<i>B. paraptussis</i> 70kD outer-membrane protein.

<i>rhI</i>	Repressor gene of phase 1 flagellin gene of <i>S. typhimurium</i> .
RNA	Ribonucleic acid.
SDS	Sodium dodecyl sulphate.
SDW	Sterile distilled water.
SGM	Society for General Microbiology.
SS	Stainer and Scholte medium.
SSC	Standard saline citrate.
TBE	Tris-borate-EDTA buffer.
TE	Tris-EDTA buffer.
TES	Tris-EDTA-salt buffer.
Tn	Transposon.
<i>vir</i>	Virulence-regulatory gene.
<i>vrg</i>	<i>vir</i> -repressed gene.
WHO	World Health Organisation.
X-gal	5-Bromo-4-chloro-3-indoyl-beta-D-galactopyranoside.
μg	Microgram.
μl	Microlitre.
(w/v)	Weight: volume ratio.
(v/v)	Volume: volume ratio.
°C	Degrees Celsius.
³² P	Radioisotope of phosphorus.
³⁵ S	Radioisotope of sulphur.

CODES FOR AMINO ACIDS.

	Three Letter Abbreviation	One Letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or Aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or Glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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CHAPTER 1

GENERAL INTRODUCTION.

1.1. THE GENUS *BORDETELLA*.

The genus *Bordetella* is comprised of four species, all of which are respiratory tract pathogens: *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*. *B. pertussis* and *B. parapertussis* are human pathogens while *B. bronchiseptica* is a broad host-range mammalian pathogen and *B. avium* an avian pathogen. *B. pertussis* causes pertussis or whooping cough in humans. Originally *B. pertussis* was classified in the genus *Haemophilus* due to an apparent requirement for blood in the culture medium. Further work showed that blood was not a nutritional requirement but its presence aided in absorbing growth inhibitors (Hornibrook, 1940). The bordetellae were therefore subsequently named after Jules Bordet who in 1906 with O. Gengou first isolated the Gram-negative coccobacillus now known as *B. pertussis*. The name pertussis was derived from the Latin (per = severe, tussis = cough) relating to the symptoms of whooping cough in man. *B. parapertussis* was distinguished as a separate species by Eldering and Kendrick (1938) and is generally regarded as causing a milder form of pertussis (Lautrop, 1971). In addition to infecting humans, it has also been isolated from healthy and pneumonic sheep (Chen *et al.*, 1988).

B. bronchiseptica was first isolated from the respiratory tracts of dogs (Ferry, 1911). It has been found to be the primary cause of kennel cough in dogs (Wright *et al.*, 1973) and one of the agents of atrophic rhinitis in pigs (Rutter, 1983). It has also been shown to be a respiratory tract pathogen of numerous laboratory and domestic animals. It can be encountered occasionally as a commensal in the human respiratory tract and, rarely, as a pathogen in immunocompromised patients (Woolfrey and Moody, 1991). The biology of *B. bronchiseptica* has been reviewed by Goodnow (1980). *B. avium* is an agent of respiratory disease of poultry and in particular turkey coryza or rhinotracheitis (Kersters *et al.*, 1984). Although it causes a severe respiratory disease in turkeys, it shows a mild to unapparent infection in other avian species. The species had been previously identified as "bronchiseptica-like", then *Alcaligenes faecalis*

(Hinz *et al.* 1978; Simmons *et al.*, 1980) until Kersters *et al.* (1984) placed it in the bordetellae.

A close phenotypic and genetic relationship between the four species has been shown on the basis of serological characteristics and by DNA-rRNA hybridisation studies (Kersters *et al.*, 1984), DNA-DNA hybridisation of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* (Kloos *et al.*, 1981) and electrophoretic mobility patterns of metabolic enzymes (Musser *et al.*, 1986). *B. avium* has a lower G + C content (61.6-62.6 mol%) compared to the other bordetellae (67.7-69.5 mol%).

1.2. PERTUSSIS-THE DISEASE.

All human age groups are susceptible to pertussis or whooping cough, but the most clinically severe cases are seen in babies under one year old. Following infection with *B. pertussis*, or occasionally *B. parapertussis*, the classical disease syndrome has three notable stages: catarrhal, paroxysmal and convalescent. After an incubation period of 6-20 days the "catarrhal stage" develops with mild coughing and sneezing resembling a common cold. This stage may last several weeks, during which *B. pertussis* can be isolated from the patient. The bacteria adhere to the epithelial cells of the respiratory tract resulting in ciliostasis and shedding of the ciliated cells causing the accumulation of thick mucus. During the paroxysmal stage the cough develops its explosive character and the characteristic whoop, which is caused by inhalation past the swollen, partially closed glottis. The violent coughing serves to clear the airways of accumulated mucus and cell debris. The paroxysmal stage usually lasts between 1-4 weeks, then the symptoms slowly begin to decrease as the patient enters the convalescent phase which can last for 10-16 weeks (Walker, 1988).

Many of the complications associated with the disease are the result of the force of the coughing fits. Serious sequelae such as seizures, encephelopathy and death can occur after the paroxysmal stage and secondary infections can give

rise to bronchial pneumonia (Jamieson, 1973). Pertussis and sequelae have been reviewed by Olson (1975) and Manclark and Cowell (1984). The isolation rate for *B. pertussis* drops progressively after the onset of the paroxysmal phase. Broad spectrum antibiotics given during the early stage of the disease are effective against the bacteria but have little or no effect on the clinical course of the established disease. Fig. 1.1. shows a diagrammatic representation of the underlying sequence of events in pertussis. *B. bronchiseptica* and *B. avium* also cause localised infections of the upper respiratory tract in their respective hosts. The diseases have characteristics in common with each other and with whooping cough in humans (Gray *et al.*, 1983).

1.3. GROWTH OF THE BORDETELLAE.

Bordet-Gengou agar or charcoal agar are used as solid media for the growth of bordetellae. Additions to the media of blood, charcoal, soluble starch or anionic-exchange resins absorb growth inhibitors. A chemically-defined medium (Stainer and Scholte, 1971) with the addition of heptakis (2, 6-0-dimethyl) beta cyclodextrin (Imaizumi *et al.*, 1983) is suitable for broth cultures. Growth conditions and media have been reviewed by Stainer (1988). *B. paraptussis*, *B. bronchiseptica* and *B. avium* in contrast to *B. pertussis*, are not sensitive to the growth inhibitors in solid media and can be successfully grown on nutrient agar.

1.4. VIRULENCE FACTORS OF *B. PERTUSSIS*.

B. pertussis produces a wide variety of virulence-associated factors and adhesins. These have been assigned roles in the disease process, promoting a fuller understanding of the pathogenicity of pertussis. Virulence determinants have been reviewed by Wardlaw and Parton (1983), Robinson *et al.* (1985a), Weiss and Hewlett (1986) and Wardlaw and Parton (1988). It was originally thought that the symptoms of pertussis were mediated by pertussis toxin (PT)

Fig. 1.1. Diagrammatic representation of the clinical stages in pertussis and the underlying sequence of events (redrawn from Parton, 1989).

CLINICAL STAGES IN THE HOST

EXPOSURE



INCUBATION



CATARRHAL



PAROXYSMAL



RESPIRATORY AND
CNS COMPLICATIONS

CONVALESCENT

PARASITE EVENTS

TRANSMISSION OF *B. PERTUSSIS* TO

SUSCEPTIBLE HOST



ATTACHMENT AND GROWTH



SECRETION OR RELEASE OF TOXINS



STIMULATION OF HOST
IMMUNE RESPONSES AND
REPAIR MECHANISMS



TERMINATION OF
INFECTION



RECOVERY FROM DISEASE



IMPAIRMENT OF
HOST DEFENCES;
INITIATION OF
LOCAL AND
SYSTEMIC
PATHOLOGY



alone with the aid of several adhesins (Pittman, 1979). The inability to isolate the organism during the most severe stage of the disease led to the hypothesis that the majority of clinical symptoms were due to a long-acting toxin(s) produced by the bacteria (Pittman, 1984b). Subsequent studies have revealed a larger number of virulence-associated factors which appear to play a role in *B. pertussis* infection. The construction of a number of *B. pertussis* strains containing single Tn5 insertions within genes encoding various virulence factors (Weiss *et al.*, 1983) has played a major role in assessing the contribution of individual factors to the disease in laboratory animals. For example the virulence properties of a mutant strain lacking a given factor have been compared to those of the wild type strain in an infant mouse model (Weiss *et al.*, 1984).

Attachment of the *Bordetella* to host tissue is a first step in pathogenesis. In whooping cough, several bacterial factors have been implicated. These include pertussis toxin; filamentous haemagglutinin, FHA, (Tuomanen and Weiss, 1985); fimbriae (Mooi *et al.*, 1992) and a 69 kD outer-membrane protein, pertactin, (Leininger *et al.*, 1991). Toxins are also important in the infectious process and include pertussis toxin and adenylate cyclase toxin. Further toxins have also been shown to be produced by *B. pertussis*: lipopolysaccharide (endotoxin), heat-labile dermonecrotising toxin and tracheal cytotoxin. All the *Bordetella* species share certain virulence factors, which include the dermonecrotizing toxin and tracheal cytotoxin (Gentry-Weeks *et al.*, 1988), but *B. avium* lacks the genes for agglutinogens, adenylate cyclase toxin and pertussis toxin present in the other three species (Mooi *et al.*, 1987; Arico and Rappuoli, 1987; Brownlie *et al.*, 1988). Although *B. pertussis* is the only *Bordetella* to produce pertussis toxin (PT), *B. parapertussis* and *B. bronchiseptica* have been shown to contain mutated, transcriptionally-silent PT genes (Arico *et al.*, 1987). Motility is expressed only by *B. bronchiseptica* and *B. avium* (Pittman, 1984a; Kersters *et al.*, 1984). The various virulence factors of *B. pertussis* will now be examined in more detail.

1.4.1. Pertussis toxin.

Although the actual role of pertussis toxin (PT) in whooping cough is far from clear, there has been much speculation. The toxin has had many names, reflecting its wide range of biological activities. These include lymphocytosis-promoting factor (LPF), histamine-sensitising factor (HSF), pertussigen and islets-activating protein (IAP) (Yajima *et al.*, 1978). It has been hypothesised (Pittman, 1979; 1984b) as being responsible for the paroxysmal cough and therefore of fundamental importance in the pathogenesis of pertussis. The adhesion properties of PT have been shown in mutation experiments. Transposon mutants lacking PT show decreased adherence to rabbit and human ciliated cells in an *in vitro* assay (Tuomanen and Weiss, 1985). Purified PT is capable of conferring protection to mice against intranasal and intracerebral challenge with *B. pertussis* (Munoz *et al.*, 1981). PT and its effects have been widely reviewed, by Wardlaw and Parton (1983), Sekura *et al.* (1985), Ui (1988), Furman *et al.* (1988), Munoz (1988), Monack *et al.* (1989b) and Kaslow and Burns (1992).

The role of PT has been better understood since its structure was determined. PT is composed of six polypeptides of five different types, S1 - S5 . An A - B subunit structure has been proposed for the toxin, the A subunit being the active part, the B subunit the binding oligomer (Tamura *et al.*, 1982). The A or S1 subunit is enzymatically-active causing ADP-ribosylation of a G protein, G_i in target mammalian cells. G_i is a guanine nucleotide-binding protein that inhibits adenylate cyclase from generating cyclic AMP (Yajima *et al.*, 1978; Katada and Ui, 1982) and ADP-ribosylation prevents this and thus disrupts normal cell function by raising internal cAMP concentrations. The B subunit oligomer is composed of the polypeptides S2 to S5 in the ratio 1:1:2:1 (Katada and Ui, 1982) and consists of two dimers (S2-S4 and S3-S4) connected by S5. The B oligomer binds to the target cell receptors of eukaryotic cells facilitating

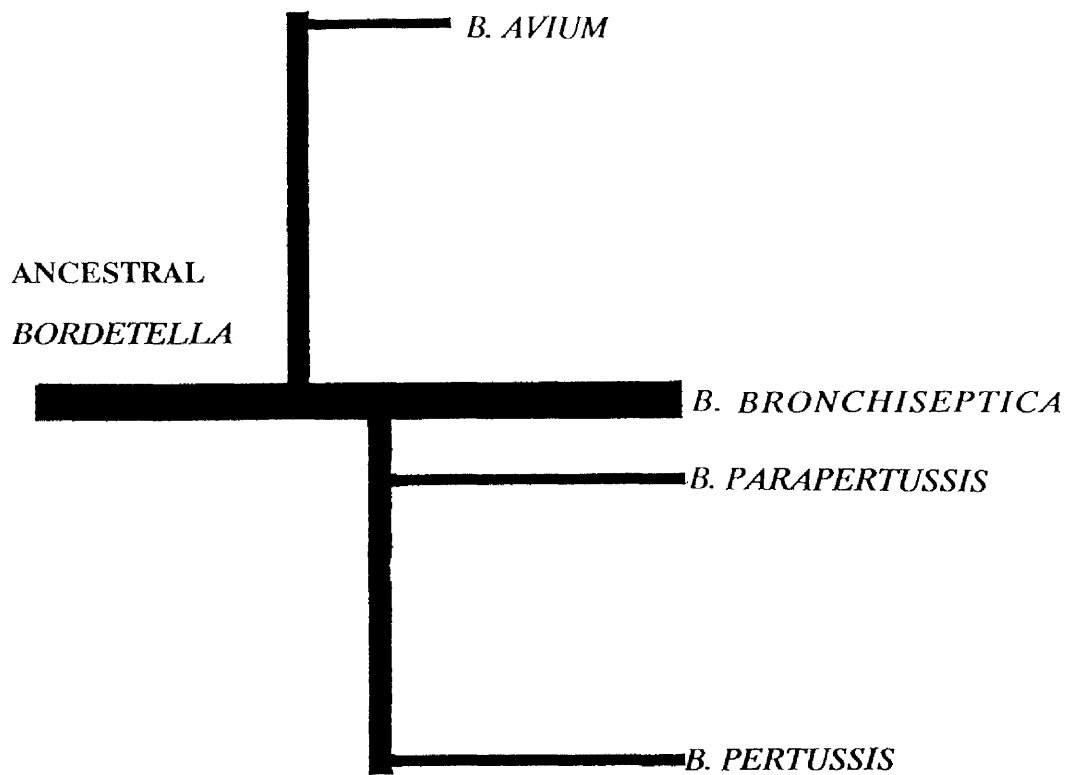
the translocation of the S1 subunit across the membrane and into the cells by receptor-mediated endocytosis (Tamura *et al.*, 1982, Burns, 1988). *B. pertussis* binds to glycoconjugates on human cilia and macrophages; the results of Wout *et al.* (1992) indicate that the carbohydrate recognition domains of both S2 and S3 participate in adherence of *B. pertussis* to human macrophages.

The PT genes have been cloned (Locht *et al.*, 1986; Nicosia *et al.*, 1986) and sequenced, and the amino-acid sequences of the individual subunits deduced. The amino acid sequences of S2 and S3 were found to be 70% homologous. The genes are arranged in an operon under the control of a single positively-regulated promoter. The PT genes present in *B. bronchiseptica* and *B. parapertussis* display a high (over 96%) degree of nucleotide sequence homology to those in *B. pertussis* but neither of the former species expresses the genes (Arico and Rappuoli, 1987). This is probably due to multiple mutations in their promoter regions (Marchitto *et al.*, 1987). The cloned PT gene from *B. pertussis* is expressed in both of these species (Lee *et al.*, 1989) but PT is not secreted, suggesting that their PT transport mechanisms are either absent or non-functional. No DNA homology to PT genes has been shown in *B. avium*.

Arico *et al.* (1987) and Gross *et al.* (1989) have discussed the possible evolutionary relatedness of the bordetellae based on the nucleotide sequence of the pertussis toxin operon (Fig. 1.2). This figure shows *B. avium* as the most distant relative of *B. pertussis* as PT genes are not present. *B. parapertussis* and *B. bronchiseptica* are grouped together in having, albeit inactive, toxin genes. *B. pertussis* with a fully functional toxin gene is placed separately. It is not clear at present if the PT genes were acquired by the ancestral *Bordetella* strain in an active form which then mutated to inactivity through niche specialisation or vice versa.

Downstream of the PT operon are open reading frames involved in pertussis toxin biosynthesis. They have striking sequence similarities to the *virB* operons required for the transfer of the T-DNA from the resident Ti plasmid in

Fig. 1.2. Phylogenetic tree of the genus *Bordetella* deduced from a computer analysis of the nucleotide sequence of the pertussis toxin operon (Arico *et al.*, 1987; redrawn from Gross *et al.*, 1989).



Agrobacterium tumefaciens to plant cells (Weiss *et al.*, 1993; Shirasu and Kado, 1993). One of the PT open reading frames has been shown to be required for pertussis toxin secretion across the *B. pertussis* outer cell membrane (Covacci and Rappuoli, 1993).

Murine monoclonal antibodies (MAbs) against the different PT subunits have been used to define the main structural and functional domains of PT (Sato *et al.*, 1984; Sato *et al.*, 1987). Amino acid substitutions within the enzymatically-active subunit S1 abolished its ADP-ribosyl transferase activity and the genetically toxoided PT molecule protected mice from intracerebral challenge with virulent *B. pertussis* (Pizza *et al.*, 1989).

The five genes encoding pertussis toxin have been expressed separately in *E. coli* (Burnette *et al.*, 1988) but the recombinant products did not assemble to form the pertussis holotoxin product. Recombinant pertussis toxin subunits produced in *B. parapertussis* and *B. bronchiseptica* are, however, assembled as native holotoxin (Lee *et al.*, 1989). Burnette *et al.* (1992) have assembled the PT B oligomer *in vitro* after producing the recombinant polypeptides in *E. coli*. This enabled B oligomer production without active PT and other toxic constituents of the *B. pertussis* bacterium. Immunisation of mice with the recombinant B oligomer protected them from the lymphocytosis-promoting activities of PT.

1.4.2. Filamentous haemagglutinin.

Filamentous haemagglutinin, FHA, is a protein located on the surface of *B. pertussis* and is also secreted into the culture medium during growth. The protein forms filamentous aggregates 2 nm in diameter and 40-100 nm long (Arai and Sato, 1976). Haemagglutination by *B. pertussis* was first reported by Keogh *et al.* (1947). Two haemagglutinins, FHA and PT are now recognised (Arai and Sato, 1976). FHA is 5-7 times more active as a haemagglutinin than PT but is sensitive to inhibition by micromolar concentrations of cholesterol (Sato *et al.*, 1983).

Antibodies against both haemagglutinins have been shown to confer passive protection against intranasal challenge in mice (Munoz *et al.*, 1981), suggesting a role for these factors in pathogenesis. The purified FHA molecule confers active immunity against aerosol challenge in mice (Kimura *et al.*, 1990). These authors also indicated that FHA is an important factor in the initial adherence and colonisation of the trachea. Observations supporting an adhesive role for PT and FHA have been reported by Tuomanen (1986). Transposon mutants lacking FHA show decreased adherence to rabbit and human ciliated cells in an *in vitro* assay (Tuomanen *et al.*, 1985). The addition of exogenous purified FHA to the mutant strain allowed the recovery of full adherence (Tuomanen and Weiss, 1985).

Two different binding domains for cellular attachment of FHA have been identified. One is the putative cell-binding amino acid sequence arginine-glycine-aspartic acid (RGD) (Ruoslahti and Pierschbacher, 1986). These sequences in FHA interact with receptors on macrophages (Relman *et al.*, 1990). The other binding activity is shown by the inhibition of binding of FHA to epithelial cells by the addition of galactose (Tuomanen *et al.*, 1988). A potential lectin-like site has been identified on FHA (Delisse-Gathoye *et al.*, 1990). Attachment to cells is often a prerequisite for bacteria to enter cells and virulent *B. pertussis* has been shown to enter human epithelial cells *in vitro* (Ewanowich *et al.*, 1989). In addition, bacteria have been visualised within alveolar macrophages in a mouse model of whooping cough (Cheers and Gray, 1969).

The FHA structural gene (*fhaB*) has been sequenced; the primary translation product is a protein of 370 kD which is processed to form a 220 kD FHA polypeptide. This probably occurs by proteolytic processing eliminating the carboxy-terminal portion (Delisse-Gathoye *et al.*, 1990; Domenighini *et al.*, 1990). The *fhaB* gene lies adjacent to the *bvg* locus, which encodes the components of a trans-acting regulatory system (Stibitz *et al.*, 1988; Relman *et*

al., 1989; see Section 1.15.). The *bvg* system controls the expression of FHA at the level of transcription (Miller *et al.*, 1989). Loch *et al.* (1992) identified accessory genes downstream of *fhaB* with sequence similarity to genes essential for synthesis of pili in Gram-negative organisms and mutational inactivation of these accessory genes results in a FHA-negative phenotype. Thus Loch *et al.* (1992) suggested that *B. pertussis* possesses common accessory genes responsible for the biogenesis of two different surface structures, FHA and pili.

1.4.3. Agglutinogens.

Agglutinogens (AGGs) are surface antigens that stimulate the production of agglutinins, antibodies resulting in bacterial cell agglutination. The combinations of AGGs differentiate the main *B. pertussis* serotypes, by a serotyping scheme developed by Eldering *et al.* (1957). There are six *B. pertussis* specific AGGs, of which AGG 1 is common to all strains and AGGs 2-6 can be found in various combinations on different strains. It has been suggested that the three major AGGs are 1, 2 and 3, while 4, 5 and 6 are minor ones (Preston *et al.*, 1982). AGG 7 is common to all strains of the genus *Bordetella*, while AGG 12 is specific for *B. bronchiseptica* and AGG 14 is specific for *B. parapertussis* (Robinson *et al.*, 1989a). *B. avium* shares several AGGs with *B. bronchiseptica* (Kerstens *et al.*, 1984).

The nature of AGG 1 present on all strains of *B. pertussis* has yet to be determined but it does not appear to be a fimbrial protein. Certain monoclonal antibodies that react with lipooligosaccharide A (LOS A) can agglutinate most strains of *B. pertussis*, but not *B. bronchiseptica* or *B. parapertussis* strains. Hence the polysaccharide chain of LOS has similar properties to AGG 1 (Li *et al.*, 1988). AGG 1 may be relevant for distinguishing *B. pertussis* from other *Bordetella* species (Robinson *et al.*, 1989a). The two main serotype AGGs of *B. pertussis* correspond to fimbrial components (Ashworth *et al.*, 1982; Mooi *et al.*, 1987). One type of fimbriae bears AGG 2 with a subunit Mr of 22500 and is

found on 1,2,0 and 1,2,3 serotypes. Another type bears agglutinin 3 with a subunit Mr of 22000 and is found on 1,0,3 and 1,2,3 serotypes (Robinson, 1989b). The amino acid sequences of the fimbrial AGG2 and 3 subunit proteins are very closely related (Mooi *et al.*, 1987). The amino-terminal sequences of these subunits have also been shown to share 50% homology with that of *E. coli* K99 fimbrial subunits (Mooi *et al.*, 1987).

The fimbrial agglutinogens of *B. pertussis* have been suggested to act as adhesins to the ciliated epithelial cells of the respiratory tract during the disease process. This is by analogy with other bacterial pathogens where adherence is associated with possession of fimbriae (de Graaf and Mooi, 1986). Monoclonal antibodies to AGGs 2 and 3 inhibit the binding of *B. pertussis* to Vero (monkey kidney) cells and HeLa cells but these are non-ciliated (Gorringe *et al.*, 1985; Redhead, 1985). Tuomanen and Weiss (1985) have shown that non-fimbriate *B. pertussis* adhere to ciliated human cells. This resulted in Tuomanen (1988) suggesting that there is little evidence for the role of AGGs as adhesins to ciliated cells in humans, although cell-types or receptors to which *B. pertussis* fimbriae adhere specifically may yet be identified. To study the relevance of *B. pertussis* fimbriae to bacterial disease, fimbrial mutants were constructed by Mooi *et al.* (1992). A fimbrial mutant was analysed in a mouse respiratory infection model together with a strain harbouring a deletion in the gene for FHA. Both mutants were affected in their ability to persist in the trachea. Persistence in the nasopharynx was affected only by the mutation in the FHA gene. Neither the FHA nor the fimbrial mutants were affected in their ability to persist in the lung.

Although these results suggest that the *B. pertussis* fimbriae may not be adhesins to human ciliated respiratory cells, their ability to produce an antibody response is important in discussions on vaccine production. Mice immunised with purified fimbriae are protected against subsequent intranasal challenge with virulent *B. pertussis* (Robinson *et al.*, 1985b; Zhang *et al.*, 1985). Protection

afforded by purified fimbrial AGGs is also found to be serospecific (Ashworth *et al.*, 1988; Robinson *et al.*, 1989b).

The two genes encoding the AGG 2 and 3 fimbrial subunits (*fim2* and *fim3* respectively) have been cloned and sequenced (Livey *et al.*, 1987; Mooi *et al.*, 1990). Pedroni *et al.* (1988) have detected in *B. pertussis* a gene designated *fimX* which shows homology in its nucleotide sequence with the genes coding for fimbrial AGGs 2 and 3. Riboli *et al.* (1991) have shown the *fimX* promoter to be active, although at a low level and to be *vir*-regulated. Thus the *fimX* protein might be produced in minute quantities which are not detectable by conventional methods. It is possible that FimX (20kD), an as yet unidentified polypeptide, could be a structural component of *B. pertussis* fimbriae of a different serotype. The work of Mooi *et al.* (1992) on fimbrial mutants has suggested the presence of a fourth fimbrial gene, *fimY*.

TnphoA mutagenesis by Willems *et al.* (1992) identified three additional fimbrial genes designated *fimB*, *fimC* and *fimD*. The fimbrial gene cluster was positively regulated by the *bvg* locus. Interestingly the fimbrial gene cluster was found to be located directly downstream of the gene coding for FHA (Locht *et al.*, 1992). The close association of the *fim* and *fha* genes suggests co-operation between the two loci in the pathogenesis of pertussis or in mutual aspects of assembly of the components of the cell surface.

The structural gene of the serotype 2 fimbrial subunit has been expressed in *E. coli* (Walker *et al.*, 1990). However, it is antigenically different from that produced in *B. pertussis* and exhibits only limited cross-reactivity to wild-type fimbriae. This suggests that assembly of fully immunogenic fimbriae in *E. coli* may require additional fimbrial assembly proteins or minor fimbrial structural proteins. These could well be the gene products of *fimB,C* and *D* (Willems *et al.*, 1992). Walker *et al.* (1991) demonstrated that recombinant serotype 2 fimbrial subunits that are indistinguishable from native fimbriae can be produced in *B. paraptussis* and *B. bronchiseptica* expressing fimbrial accessory genes.

1.4.4. Outer-membrane proteins.

The 69 kD non-fimbrial outer-membrane protein (P69) is an agglutinin found on all virulent strains of *B. pertussis* regardless of serotype (Brennan *et al.*, 1988). Antigenically -related proteins have also been found in *B. parapertussis*, P70 (70 kD), and *B. bronchiseptica*, P68 (68 kD). The genes encoding P69, P70 and P68 have been cloned and sequenced (Charles *et al.*, 1989; Li *et al.*, 1991a; 1992). Slide agglutination of *E. coli* expressing P70 and P68 with anti-P69 antibody, strongly suggest that these proteins also can function as agglutinogens (Li *et al.*, 1991a; 1992). The name pertactin (per = pertussis and tactin = to touch) has been proposed for P69 because of the ability of the protein to bind to eukaryotic cells (Leininger *et al.*, 1991). Like FHA, pertactin (69kD) contains the putative cell-binding amino acid sequence RGD (Charles *et al.*, 1989). This suggests that P69 could have a role in bacterial adhesion. Two apparent isoforms of the virulence-associated 69 kD protein have been purified and examined for structural and immunological differences (Gotto *et al.*, 1993). Mass spectrometry showed a difference of 2,060 Da; both forms were protective in a mouse model, eliciting bactericidal antibodies and reducing respiratory tract colonisation.

1.4.5. Lipopolysaccharide endotoxin.

As with other Gram-negative bacteria, lipopolysaccharides are produced by all the bordetellae and have the characteristic range of endotoxin activities (Chaby and Caroff, 1988). *B. pertussis* produces two distinct lipopolysaccharides, LPS-I and LPS-II (Peppler, 1984). In common with LPS from other bacteria, they are components of the cell wall, heat-stable, antigenic, pyrogenic and toxic. The occurrence of anti-LPS in pertussis convalescent sera has been described by Winsnes (1988) but its role in pertussis pathogenesis has not been established. SDS-PAGE profiles have indicated that the LPS of some *B. pertussis* strains

lacked high molecular weight bands suggesting a lack of long O-side chains (Byrd *et al.*, 1991). Other workers have shown that LPS profiles of *B. pertussis* strains contain intermediate to small molecular weight bands typical of O-side chain material. This later type has been referred to as lipooligosaccharide or LOS (Li *et al.*, 1988). As previously mentioned, Li *et al.* (1988) have presented evidence suggesting that LOS A may correspond to AGG-1. *B. parapertussis* and smooth strains of *B. bronchiseptica* had LPS profiles with similar high mol. wt bands which were interpreted as core structure fragments with variable length O-side chain-like material (Byrd *et al.*, 1991).

1.4.6. Adenylate cyclase toxin and haemolysin.

Hewlett and Wolff (1976) discovered that *B. pertussis* produces adenylate cyclase, an enzyme that converts ATP to cyclic AMP (cAMP). The cytotoxic form of adenylate cyclase (ACT) is able to enter eukaryotic cells. This generates high levels of intracellular cAMP, resulting in impaired cellular function (Confer and Eaton, 1982; Middlebrook and Dorland, 1984). Weiss *et al.* (1983) isolated a mutant with a single Tn5 insertion which had lost both adenylate cyclase toxin and haemolytic activities. Evidence for the genetic relationship between the two activities was confirmed by Brownlie *et al.* (1986) using a recombinant cosmid which restored AC and HLY activity to this mutant. Glaser *et al.* (1988) cloned and expressed the adenylate cyclase operon. The sequence data revealed the adenylate cyclase operon to be composed of four open reading frames, *cyaA*, *B*, *D* and *E* (Glaser *et al.*, 1988). The genes *cyaB*, *D* and *E* are located downstream from the *cyaA* gene and their gene products are necessary for transport of the toxin from the cell. The *cyaA* gene encodes the virulence factor, cyclolysin, a bifunctional protein of predicted Mr 177kD exhibiting adenylate cyclase and haemolytic activities. The toxin is activated by calmodulin, a eukaryotic calcium-binding protein (Wolff *et al.*, 1980) and the 400-residue amino-terminal domain of *cyaA* corresponds to the calmodulin (CaM)-activated catalytic activity.

The carboxy-terminal 1,300 residues of the *B. pertussis* structural gene *cyaA* were found to show significant similarity with the *E. coli* structural gene, *hlyA*, for alpha haemolysin. In addition, the *cyaB* and *D* genes are homologous to the *E. coli hlyB* and *D* genes necessary for haemolysin secretion (Glaser *et al.*, 1988). The open reading frame *cyaE* was found to have no homologous counterpart in the *E. coli* haemolysin operon. However, subsequent analysis of an open reading frame upstream of the *cyaA* gene identified the *cyaC* gene with homology to the *E. coli hlyC* gene. It also appears to encode a protein with a function similar to that of the *E. coli* product i.e. post-translational activation of the structural gene product. The *cyaC* gene is orientated opposite to *cyaA* in contrast to *E. coli*. The difference in orientation of activator and structural genes may represent a mechanism of regulation in *B. pertussis* (Barry *et al.*, 1991).

Weiss and Hewlett (1986) showed the toxin to have a remarkably diverse spectrum of inhibitory actions on immune effector cells. The transposon mutant of *B. pertussis* unable to express the adenylate cyclase operon has been shown to have reduced virulence in mice (Weiss *et al.*, 1983; Olander *et al.*, 1986; Brownlie *et al.*, 1988; Khelef *et al.*, 1992). Monoclonal anti-ACT antibody, incubated with a challenge inoculum, protected mice from respiratory infection (Brezin *et al.*, 1987). Guiso *et al.* (1991) showed that active immunisation with purified adenylate-cyclase-haemolysin (AC-Hly) induced a protection in mice against *B. pertussis* significantly shortening the period of bacterial colonisation of the mouse respiratory tract. These data indicate that the ACT is an important virulence factor and may be an important immunogen.

1.4.7. Dermonecrotic toxin.

Demonecrotic toxin (DNT) or heat-labile toxin (HLT) is produced by *B. pertussis*, *B. paraptussis* and *B. bronchiseptica*. The toxin causes necrotic lesions in mice when injected subcutaneously at low doses, and is lethal if the dose is increased (Livey and Wardlaw, 1984). It has a constrictive effect on vascular

smooth muscle and may cause inflammation of the respiratory tract mucosa during the whooping cough disease process (Nakase and Endoh, 1988) but its exact role in the disease process has not been defined.

1.4.8. Tracheal cytotoxin.

The isolation and determination of the mode of action of tracheal cytotoxin (TCT) was done by Goldman *et al.* (1982). Cookson *et al.* (1989) have proposed that TCT is derived by enzymatic cleavage of the cell wall component peptidoglycan. TCT has been shown to have cytopathic effects on cells of the respiratory epithelium of hamster tracheal rings *in vitro* (Goldman and Herwaldt, 1985). Damage to, and the subsequent extrusion of ciliated cells from, the respiratory epithelium in *in vitro* studies resembles the histopathological events in the disease process of pertussis. All four *Bordetella* species produce TCT.

The locations of the genes encoding various virulence factors in the chromosome of *B. pertussis* are shown in Fig. 1.3.

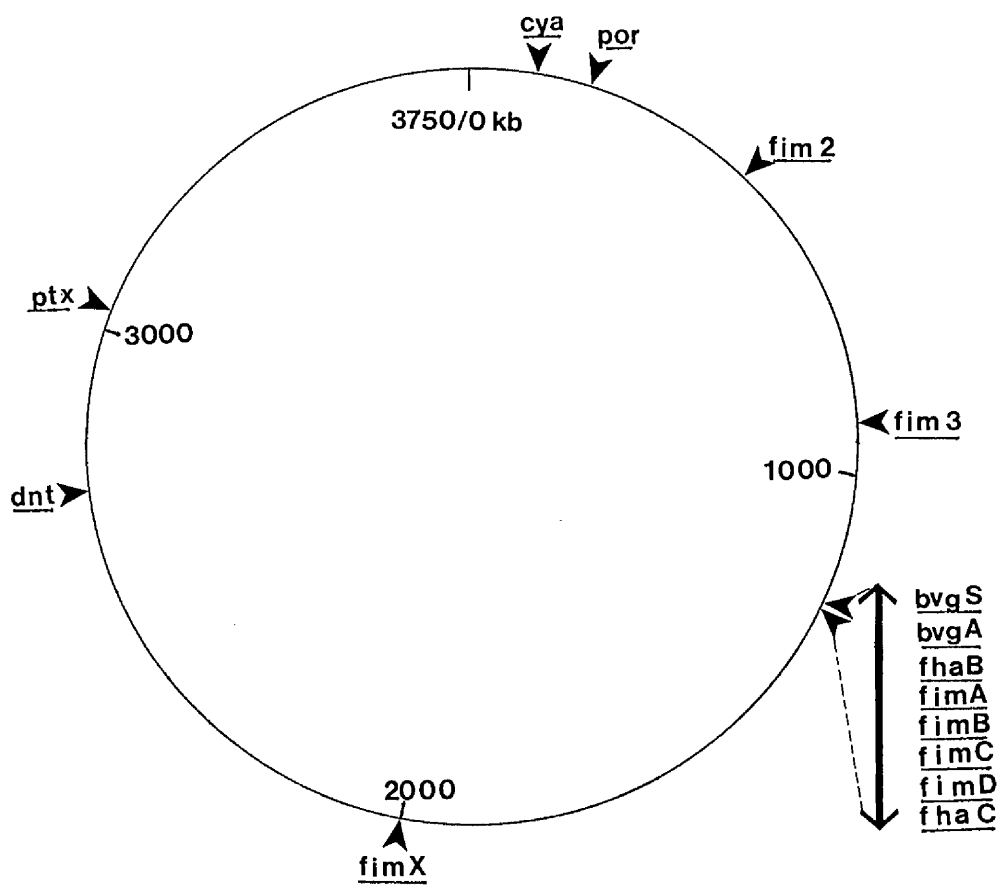
1.5. FLAGELLA IN BORDETELLA AND OTHER ORGANISMS.

The potential of bacterial flagella as virulence factors derives from their capacity to promote motility, propelling bacteria towards a chemoattractant or aiding penetration of the mucous blanket and epithelial cells (Poxton and Arbuthnott, 1990). Flagella are putative virulence factors for *Vibrio cholerae* enabling penetration of the mucous blanket of the small intestine (Attridge and Rowley, 1983). Historically *Bordetella* flagella have not been regarded as virulence factors, and so are dealt with in a separate section, although recently there has been more interest in these flagella and their manner of expression. Flagella and motility are expressed in only two of the *Bordetella* strains: *B. bronchiseptica* and *B. avium* (Pittman, 1984a; Kersters *et al.*, 1984) and the flagella are in a peritrichous arrangement. They are not expressed in the human

Fig. 1.3. The location of genes (arrowed) encoding various virulence factors in the chromosome of *B. pertussis* shown diagrammatically.

Coordinates are given in kilobase pairs. (Redrawn from Stibitz and Garletts, 1992).

Abbreviations: *cya*: adenylate cyclase (or cyclolysin) gene; *por*: porin gene; *fim2*, *fim3*, *fimX*, *fimA*, *B*, *C*, *D*: fimbrial genes; *fhaB*, *fhaC*: filamentous haemagglutinin genes; *bvgS*, *bvgA*: *Bordetella* virulence genes; *dnt*: dermonecrotic toxin gene and *ptx*: pertussis toxin gene.



pathogens, *B. pertussis* or *B. parapertussis*. *B. bronchiseptica* produces two classes of flagellins of 35 and 40kD but only one type is expressed by any one strain. All *B. avium* strains tested by Akerley *et al.* (1992) expressed only a 42kD flagellin. Amino acid sequence analysis of the two *B. bronchiseptica* flagellins at their N-terminal regions revealed 100% identity with each other and 80% identity with *S. typhimurium* flagellin. The extensive conservation of flagellin sequences at the amino and carboxy termini has been noted for a variety of genera (Joys, 1988) and it has been proposed that these domains are essential for filament formation (Kuwajima, 1988).

In both *S. typhimurium* and *E. coli*, which encode flagellin molecules that are similar to those expressed by *B. bronchiseptica*, hierarchical control of flagellar component synthesis has been demonstrated (Komeda, 1982; Macnab, 1987; Kutsukake *et al.*, 1990; Gillen and Hughes, 1991). The flagellar genes of *S. typhimurium* and *E. coli* are found in clusters termed regions I, II and III in the chromosome. Within each region there are several operons each containing anywhere from one to as many as nine genes. The flagellar operons form a coordinated system of expression, a regulon. Four classes have been defined (1, 2, 3a and 3b) where expression of one operon in a given class is necessary for expression of subsequent classes. The regulatory hierarchy acts to ensure that the highly expressed filament structural protein, flagellin, is synthesised only after a prerequisite set of other structural proteins has been expressed and properly assembled. Kutsukake *et al.* (1990) provided evidence that the cascade transcription of the flagellar regulon closely parallels the assembly hierarchy of the flagellar structure. They proposed that classes 1, 2, and 3 be called the early, middle, and late flagellar operons. The flagellar operons from enteric bacteria are preceded by a DNA sequence highly homologous to *B. subtilis* (σ)²⁸ promoters. This might be a means of controlling the transcription of the flagellar genes (secondary, alternative sigma factors to the predominant RNA polymerase, Helmann and Chamberlain, 1987). As a further control in *S.*

typhimurium, anti-sigma factor *flgM* protein binds to the secondary sigma factor and disturbs its ability to form a complex with RNA polymerase core enzyme; a novel type of negative regulator (Ohnishi *et al.*, 1992). Transcriptional regulatory signals for flagellar genes are highly conserved between bacteria separated by a large evolutionary distance. They suggested that one possibility was that these particular functions of flagella were so essential to primordial bacterial growth that they were established early in evolution (Arnosti and Chamberlain, 1989).

1.6. PERTUSSIS VACCINE.

The pertussis vaccine currently in use in the UK is a suspension of killed whole cells. The suspension is usually administered in combination with purified toxoids of diphtheria and tetanus as the DTP vaccine. The introduction of pertussis vaccine into widespread use in the UK during the 1950s led to a marked reduction in the incidence of the disease. The use of the vaccine, however, became controversial due to certain side effects. These included minor reactions: redness, pain, fever which occur in most of the vaccinees; worrisome reactions: prolonged crying, convulsions and collapse which occurred at a frequency of 0.1-1% of recipients. In addition to these conditions, serious side effects such as brain damage and death have been associated with pertussis vaccination (Manclark and Cowell, 1984; Ross, 1988; Brennan *et al.*, 1992). Although not been shown to be due to the vaccine (Griffith, 1989), concern over these serious reactions, although rare, and the subsequent reduction in use of the vaccine due to the lack of public confidence since the mid 1970s, has encouraged work on a defined-component or acellular vaccine with fewer side effects (Robinson *et al.*, 1985a). In addition, the whole-cell vaccine fails to confer lasting immunity and may not decrease disease transmission or prevent infection, thus protecting against the disease rather than the infection.

Sato *et al.* (1984) co-purified PT and FHA, inactivated the PT with formaldehyde, and developed the first acellular vaccines which have been used

in Japan since 1981. Adverse reactions were found to be less frequent than with the whole-cell vaccine, though the children were immunised only when they had reached the age of two. To characterise further the Japanese vaccines two were tested in controlled field trials in Sweden during 1986 to 1987. One was a two component vaccine containing pertussis toxoid (PTd) and FHA (JNIH-6, National Institute of Health in Japan) and the other was a monocomponent vaccine consisting only of PTd (JNIH-7). The two-component vaccine provided better long-term protection against pertussis than the monocomponent vaccine (Storsaeter and Olin, 1992). Protection, however, appeared to be lower than that experienced with conventional whole-cell vaccines. Although it had been a double-blind trial with placebos, the whole cell vaccine had not been examined simultaneously, reliance instead being placed on historical comparisons. For a better comparison, acellular and whole-cell vaccines need to be compared at the same time (Brennan *et al.*, 1992).

A further concern was the reversion to toxicity noted with the formalin-inactivated PTd (Storsaeter and Olin, 1992). As a result, investigations have been made to genetically manipulate the PT operon to produce a non-toxic immunogen. To remove enzymatic activity, critical amino acids in the S1 subunit were identified and the gene subjected to *in vitro* site-specific mutagenesis to give codon changes. The PTd holotoxin was then assembled *in vitro* (Burnette *et al.*, 1992) or the wild-type toxin operon of *B. pertussis* was altered to produce S1 mutants by homologous recombination to create *B. pertussis* strains that secrete fully assembled non-toxic PT analogues (Rappuoli *et al.*, 1991). Both sets of analogues have been shown to protect mice from both intracerebral and aerosol challenges. Rappuoli *et al.* (1991) have tested the mutant toxin in adult volunteers, confirming its safety and high immunogenicity. In addition, after combination with diphtheria and tetanus toxoids, the products have been tested in children and infants. Initial results show that the local and systemic reactions induced by the new vaccines are reduced dramatically and that they are

antigenically superior to whole-cell vaccines (Rappuoli *et al.*, 1992b). As an alternative, the *B. pertussis* strain producing the non-toxic form of PT could be used in the form of a whole-cell vaccine, along with diphtheria and tetanus toxoids. This would induce the same immunity as the conventional vaccine and enable antibodies to other *B. pertussis* virulent factors to be stimulated (Marsili *et al.*, 1992).

The move towards an acellular vaccine has been enhanced by the identification with greater certainty of those antigens which can induce a protective immune response (Robinson *et al.*, 1985a). In the UK, an acellular vaccine composed of separately purified and formaldehyde-detoxified PT, FHA, AGG2 and AGG3 has been developed. The vaccine was protective in mice challenged intranasally with *B. pertussis*. It also produced only low reactogenicity in human adult volunteers and induced high antibody responses to FHA, PT and agglutinogens (Rutter *et al.*, 1988; Robinson and Ashworth 1988; Hambleton *et al.*, 1988).

In addition to the *B. pertussis*-specific antigens used above, pertactin (69kDa) is now also being included in some vaccines. A comparison of the serological responses to 13 acellular pertussis vaccines containing various combinations of five antigens has been made. The vaccines were given to infants at 2, 4 and 6 months. All preparations generated antibodies at levels equivalent to or greater than two licensed whole-cell products and had substantially reduced reactogenicity (Zealey *et al.*, 1992). A vaccine composed of all five of these antigens along with diphtheria and tetanus toxoids is currently being tested in Sweden, for the results to be examined in 1995 (Englund *et al.*, 1992; Zealey *et al.*, 1992). The ultimate aim is a new, defined, well-characterised, non-reactogenic, and efficacious acellular vaccine.

1.7. VARIATION IN EXPRESSION OF VIRULENCE FACTORS IN *B. PERTUSSIS*.

Establishment of a role for *B. pertussis* virulence factors in disease is complicated by the variation in their expression by several mechanisms.

1.7.1. Serotype variation.

Stanbridge and Preston (1974) observed variants of *B. pertussis* isolates which had independently lost or gained agglutinogens 2 or 3. This serotype variation was at frequencies of 10^{-3} to 10^{-4} . The phenomenon also occurs in experimental animals *in vivo* (Preston *et al.*, 1980) and in the child (Preston and Stanbridge, 1972). It is believed that serotype variation may aid survival of the pathogen in hosts which lack immunity to the new variant. Immunisation of mice with a purified fimbrial AGG exerts a selective pressure against organisms expressing the homologous AGG (Robinson *et al.*, 1989b). Work by Willems *et al.* (1990) and Mooi *et al.* (1990) indicates that serotype variation in *B. pertussis* may be due to single/small nucleotide insertion(s) or deletion(s) mutations in the cytosine-rich regulatory region upstream of the agglutinin subunit genes.

1.7.2. Antigenic modulation.

Originally the term antigenic modulation was introduced by Lacey (1960) for the phenotypic changes in the expression of *B. pertussis* virulence determinants. He reported that ionic changes in the growth environment could cause a reversible antigenic transition in *B. pertussis*. Growth on NaCl-containing media or on BG agar at 37°C produced the virulent, or X-mode phenotype, whereas growth on MgSO₄-containing media, or on BG agar at 25°C, produced the avirulent, or C-mode phenotype. Subsequently, Pusztai and Joo (1967) observed that antigenic modulation was induced by increasing the concentration of the growth factor, nicotinic acid, in the medium. During antigenic modulation,

the expression of several virulence-associated determinants of *B. pertussis* are lost: PT (Wardlaw *et al.*, 1976), FHA, HLY (Lacey, 1960), agglutinogens, AC (McPheat *et al.*, 1983), X-mode specific OMPs, X-OMPs (Parton and Wardlaw, 1975) and DNT (Livey *et al.*, 1978).

Idigbe *et al.* (1981) suggested that modulation could be explained by the selective destruction of virulence factors. However, Robinson *et al.* (1983) and Brownlie *et al.* (1985) found no inactivation of virulence factors, but rather that further synthesis was repressed. Brownlie *et al.* (1985) examined expression of PT, AC and X-OMPs during modulation. There was never an instance where one property was lost independently of the others. This is consistent with the hypothesis (Wardlaw and Parton, 1979) that a common regulatory mechanism governs the loss of these components.

1.7.3. Phase variation in *B. pertussis*.

The term phase variation was introduced for the genotypic changes in the expression of *B. pertussis* virulence determinants with laboratory passage of the bacterium (Leslie and Gardner, 1931). They described phase variation as a step-wise degradation through four distinct antigenic phases: I, fully virulent and toxic, to IV being relatively non-toxic. The phase I/phase IV classification system is still in use to denote the extremes of phase types. Weiss and Falkow (1984) introduced the more descriptive designations, "virulent phase" and "avirulent phase" as concise terms for the potential of the bacterium to cause disease when expressing all or none respectively of the virulence-associated properties. Other workers proposed events to be more complicated, with intermediate stages, in addition to the four consecutive stages (Standfast, 1951). Parker (1976, 1979) suggested that the stages were due to random accumulation of point mutations whereas Goldman *et al.* (1984) favoured an ordered multistep process. Alternatively, traits were shown to be lost and gained in a single step event (Weiss and Falkow, 1984).

During phase variation, *B. pertussis* strains lose the same virulence-associated antigens as lost during antigenic modulation. The avirulent variant produced by phase variation has an increased resistance to certain antibiotics (Bannatyne and Cheung, 1984) and to increased levels of fatty acids in the growth media (Peppler and Schrumpf, 1984), enabling it to grow on media which will not support the growth of the virulent phase organism e.g. media not supplemented with charcoal, blood, starch or cyclodextrin. Strains lose their virulence at frequencies ranging from 10^{-3} to 10^{-6} (Gross *et al.*, 1989). The transition from avirulent strain to virulent is not as freely reversible as with antigenic modulation, although revertants have been reported at low frequencies (Weiss and Falkow, 1984; Stibitz *et al.*, 1989).

1.7.4. Modulation and phase variation *in vivo*.

The avirulent phase or C-mode may represent a pathologically dormant state associated with later stages of infection (Kasuga *et al.*, 1954) and in carriers, particularly adults (Lacey 1960; Weiss and Falkow 1984). There is evidence to suggest that phase variation and antigenic modulation are not just *in vitro* artifacts (Kasuga *et al.*, 1954; Lacey, 1960). Kasuga *et al.* (1954) isolated avirulent phase organisms during the progress of the disease when patients were periodically cultured for *B. pertussis* during the course of an infection. Lacey (1960) observed that most people who were convalescent from whooping cough produced sera that would agglutinate an X-mode suspension of *B. pertussis*, but that a small percentage produced sera capable of agglutinating a C-mode suspension. These latter sera were from adults with mild coughs and nasopharyngeal swabs positive for up to 5 months. This suggested that the production of C-mode antigens was associated with growth in an environment not encountered in the acute phase of infection. Robinson *et al.* (1986) observed that C-mode cells adhere less well to mammalian cells suggesting that antigenic

modulation may aid onward transmission to other susceptible hosts during later stages of the disease. Avirulent bacteria could persist without causing disease in an infected individual, in much the same way as the normal flora persists in a human host. Such individuals could serve as a reservoir for the bacteria until a phase switch back to the virulent stage occurs and the bacteria become able to initiate new infections in susceptible hosts (Weiss and Falkow, 1984).

Virulent *B. pertussis* has the ability to invade and survive in human macrophages (Saukkonen *et al.*, 1991; Friedman *et al.*, 1992) and HeLa 229 cells (Ewanowich *et al.*, 1989). Internalisation and intracellular survival is virulence dependent (Ewanowich *et al.*, 1989, Friedman *et al.*, 1992). The intracellular population may be a reservoir from which virulent bacteria can emerge later in the course of the infection (Saukkonen *et al.*, 1991).

1.7.5. Model for modulation and phase variation in *Bordetella*.

Wardlaw and Parton (1979) hypothesised that the simultaneous loss of virulence factors during modulation and phase variation could be due to a common regulatory mechanism lying at the root of these phenomena. Weiss *et al.* (1983) demonstrated that a single Tn5 insertion caused the simultaneous loss of FHA, PT, AC, HLY, and DNT providing evidence that a single genetic locus, named *vir*, controls the expression of virulence factors. The mutant Vir⁻ strain also had decreased sensitivity to erythromycin and was comparable to the spontaneous avirulent variants selected by resistance to erythromycin. However it differed from phase IV strains as it was unable to grow on nutrient agar (Weiss and Falkow, 1984). Lax (1985) suggested that phase variation could result from random mutation in the control region, followed by selection due to the environment.

Weiss *et al.* (1983) suggested that a trans-acting function, inactivated by Tn5-insertion, was required for the expression of certain virulence factors. This was confirmed by Stibitz *et al.* (1989) using a replacement vector which, when

transferred to *B. pertussis*, enabled exchange between chromosomal DNA and homologous DNA cloned in the vector. Using this system, the genetic defect in several avirulent phase variants was mapped to the same region in the *vir* locus. This region was cloned and sequenced for both virulent and avirulent phase variants. A stretch of six cytosine bases occurred in virulent phase variants whereas in all avirulent phase variants studied this was increased to seven bases. Thus one mechanism for phase variation in *B. pertussis* would seem to be accounted for by a frame-shift mutation at an unstable stretch of cytosines in the *vir* gene (Stibitz *et al.*, 1989, Arico *et al.*, 1989). The cytosine-rich region preceding the fimbrial structural genes could have a similar function in switching on and off fimbrial gene expression. Monack *et al.* (1989a) reported that phase variants of *B. bronchiseptica* arise by spontaneous deletions (of 50-500 bp) in the *vir* locus, illustrating a further mechanism of phase variation.

Weiss and Falkow (1984) proposed that the product(s) of the *vir* gene acts as a positive effector for the expression of virulence-associated genes; turning on the transcription of many genes by interacting with their promoters. Modulation could then be due to environmental signals such as sulphate anion, nicotinic acid or low temperature influencing the expression of the *vir* locus and consequently the virulence-associated genes. The *vir* gene locus has been cloned by Brown and Parker (1987), Brownlie *et al.* (1988), Stibitz *et al.* (1988) and McGillivray *et al.* (1989). The *vir* locus has now been renamed *bvg* (*Bordetella* virulence gene) and was reported by Arico *et al.* (1989) to encode three proteins BvgA, BvgB and BvgC. Further analysis of the sequence showed only two open reading frames to be present; BvgB and BvgC were renamed BvgS. The predicted protein products of *bvgA* and *bvgS* share homology with a family of prokaryotic regulatory proteins that respond to environmental stimuli and are members of two-component sensory transduction systems. A typical signal transduction system comprises two proteins, sensor and regulator. By analogy, BvgS is a sensory transmembrane protein possibly with kinase activity and BvgA is a

transcriptional control factor that, probably when phosphorylated, activates gene expression. Environmental signals are recognised by BvgS, and then transmitted to BvgA, a positive regulator of transcription. The *bvg* locus has been shown to be structurally similar in *B. pertussis*, *B. paraptussis* and *B. bronchiseptica* (Arico *et al.*, 1991), but *B. avium* contains only DNA homologous to the *bvgS* gene (Gentry-Weeks *et al.*, 1991).

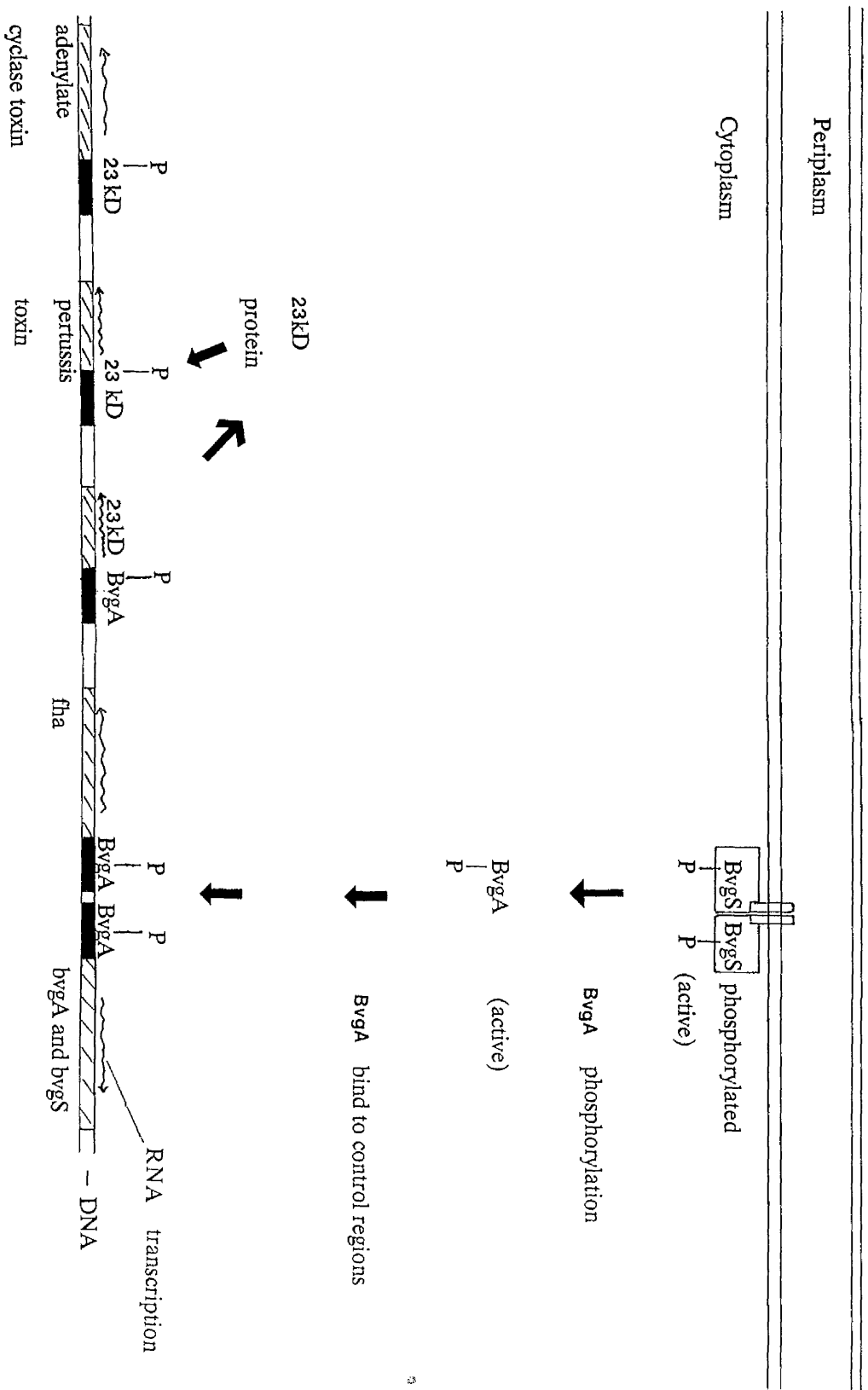
Roy *et al.* (1990) have shown that BvgA transactivates the *fhaB* gene of *B. pertussis* cloned in *E. coli*. The regulatory regions found upstream of *fhaB* and *bvgA*, involved in binding BvgA, both contain the sequence TTTCCTA. This sequence is part of an inverted repeat upstream of *fhaB* and a direct repeat upstream of *bvgA* (Roy and Falkow, 1991). Homologous sequences were not apparent upstream of other *bvg*-activated genes, such as the *ptx* operon and the *cya* operon. This suggested that the mechanism for transcriptional regulation of the other *bvg*-activated genes is complex and may require regulatory factors in addition to the *bvgAS* gene products. Gross and Rappuoli (1988) reported that two tandem 20-bp repeats upstream from the start site of transcription are necessary for pertussis toxin promoter activity and *bvg*-regulated gene expression. Huh and Weiss (1991) found two similar repeats upstream of the ACT gene but could not find them in the FHA or *bvg* promoter region. A *B. pertussis* protein was examined which specifically binds to the promoter regions of pertussis toxin and adenylate cyclase toxin genes and was found to be a 23kD protein, distinct from BvgA but regulated by the *bvg* operon. Fig. 1.4 shows a model for Bvg activation of virulence loci. Scarlato *et al.* (1990) have studied the regulation of expression of the *bvg* locus in *B. pertussis*. The control of transcription of the *bvg* locus is mediated by a 350-bp upstream sequence that contains five promoters.

Knapp and Mekalanos (1988) described a locus, *mod*, whose product was thought to act as a sensory protein for environmental signals, controlling expression of the *vir* gene. Miller *et al.* (1992) have now shown that *mod-1* is a

Fig. 1.4. Model of Bvg activation of virulence factor expression.

A dimer of BvgS phosphorylating BvgA would induce transcription of *fha* and *bvg* directly and either induce transcription (as shown here) of 23 kD locus or change the 23 kD protein from an inactive to an active state. The 23 kD protein is shown as being regulated by both transcription and phosphorylation (as speculated by Melton and Weiss (1993)). The 23kD then activates transcription of pertussis and adenylate cyclase toxin. Redrawn from Melton and Weiss (1993).

(A model of inactivation of the Bvg system is not shown. In such a model, the BvgS would be unable to dimerize and phosphorylate itself. BvgS monomers would be unable to phosphorylate BvgA, and transcription from *bvg*-regulated loci would cease.)



mutant allele of *bvgS*. Further more, Knapp and Mekalanos (1988) used *TnphoA* to detect a number of genes which were positively regulated or activated by the *vir* locus, *vir*-activated genes (*vag*) loci; and several genes which were repressed by the same mechanism, *vir*-repressed genes (*vrg*) loci. Beattie *et al.* (1992) have provided evidence by mutation that one *vrg* gene may play an important part in the virulence of *B. pertussis*, but the nature of the *vrg* gene products are at present unknown. Coordinate regulation of virulence gene expression by BvgA and BvgS appears to involve direct control at the *fhaB* and *bvgAS* promoters and indirect control of other promoters via a cascade of different trans-acting transcription factors. Thus some *vag* and *vrg* genes appear to be controlled by additional regulatory components, which in turn, are dependent on *bvgAS* for their own expression.

In *B. bronchiseptica*, motility and the synthesis of flagellins has been shown to be negatively controlled by the *bvgAS* locus (Akerley *et al.*, 1992). Environmental signals which decrease (modulate) the expression of *bvg*-activated genes lead to flagellum production and motility in *B. bronchiseptica*. Wild-type (Bvg⁺) strains are motile and produce peritrichous flagella only in the presence of modulating signals, whereas Bvg⁻ strains are motile in the absence of modulators. In *B. avium*, motility is also influenced by the modulating signals that affect virulence factor expression in other bordetellae although the effect is not as pronounced as in *B. bronchiseptica*. Nicotinic acid for example, does not induce motility (Akerley *et al.*, 1992; Jackwood *et al.*, 1991). Motility represents the first example of a phenotype found in wild-type *B. bronchiseptica* strains that is negatively-controlled by the *bvgAS*-encoded sensory apparatus. Although a regulatory link between the *bvgAS* locus and motility has been shown by Akerley *et al.* (1992), the exact mechanism of negative control remains to be established. They suggest that the active form of BvgA, resulting from phosphorylation by BvgS, binds to motility-associated promoter sequences to inhibit transcription. Modulation by

environmental signals would induce motility by decreasing the amount of active repressor. Alternatively, a more complex mechanism may be present. Scarlato *et al.* (1991) and Rappuoli *et al.* (1992a) have suggested that *B. pertussis* has evolved a system of responding to environmental changes in stages which are met first on entry into the host and then during subsequent spread throughout the respiratory tract.

1.8. FURTHER MODELS FOR VARIATION IN *BORDETELLA*.

Other mechanisms that cause variation in other bacteria have been described including DNA methylation; repeat sequences and DNA rearrangement, in particular the inversion of DNA segments.

1.8.1. DNA methylation.

E. coli contains two site-specific DNA methylases. The methylase encoded by the *dam* gene (Dam methylase) transfers a methyl group from S-adenosyl-methionine to the N⁶ position of the adenine residues in the sequences GATC (Hattman *et al.*, 1978). The other methylase is encoded by the *dcm* gene (Dcm methylase). It methylates the internal cytosine residues in the sequences CCAGG and CCTGG (May and Hattman, 1975) at the C⁵ position. These methylases are of interest here for two reasons. First, some or all of the sites for a restriction endonuclease may be resistant to cleavage when isolated from strains expressing the Dam or Dcm methylases. This occurs because methylation of particular residues within the recognition site of a restriction endonuclease will prevent cleavage, and if the methylase recognition site overlaps the endonuclease recognition site, one of these residues may be methylated. For example, DNA isolated from *dam*⁺ *E. coli* is completely resistant to cleavage by *Mbo*I, which cleaves at GATC sites.

The second reason for these methylases to be of interest is that methylation has been shown to be involved in phase variation. For example Dam

methylation controls expression of *E. coli* pyelonephritis-associated pili (Pap) where at least four proteins are involved. The DNA methylation site is located within a protein-binding domain, resulting in methylated and non-methylated DNA states that modulate gene expression by affecting regulatory protein-DNA interactions. Thus *E. coli* cells can express different types of pili which mediate their attachment to a variety of host tissues (Blyn *et al.*, 1990; van der Woude *et al.*, 1992). Norton Hughes and Johnson (1990) also found fundamental differences in DNA methylation among members of the genus *Borrelia*. Dam methylation has also been shown to affect the frequency of transposition where Tn10-promoted DNA rearrangements are more frequent in *dam*⁻ *E. coli* (Roberts *et al.*, 1985). This is the result of the absence of methylation at two GATC sites lying within the transposase gene promoter and the inner terminus of IS10. It has also been suggested that methylation might modulate the activity of other prokaryotic transposable elements such as Tn5/IS50, but not IS1 and Tn3 which transpose at equal frequencies in both *dam*⁺ and *dam*⁻ hosts and which lack *dam* methylation sites in critical regions. It has been suggested that specific growth conditions might lead to under-methylation and hence to activation of transposition. Proposed roles for Dam methylation including DNA repair and gene expression have been reviewed by Barras and Marinus, 1989.

In contrast, the biological function of 5-methyl-cytosine in CC(A/T)GG sites is not well understood. *E.coli dcm* mutants show no detectable phenotype. However, the spontaneous deamination of 5-methylcytosine results in thymine-guanine mismatches, which might give rise to mutations by C to T transitions (Coulondre *et al.*, 1978). Dcm methylation has been measured in DNA from a wide range of bacteria. In contrast to Dam methylation, which occurs in all of the family Enterobacteriaceae, and many other bacteria, Dcm methylation has been found only in those genera of the family Enterobacteriaceae closest to *Escherichia*: *Shigella*, *Citrobacter*, *Salmonella*, and *Klebsiella* (Gomez-Eichelmann *et al.*, 1991). *Bordetella* DNA was not examined in this

study.

The expression of genes in *Bordetella* could be altered by Dam and Dcm methylation to affect phase variation. The methylation state of *Bordetella* phase variants was examined by Goldman *et al.* (1987) whose results suggested that DNA of phase I *B. pertussis* strains was modified, possibly by methylation, making it resistant to certain restriction enzymes. MacGregor (1990) repeated this work but was unable to show the same variation in methylation.

1.8.2. Repeat DNA and insertion sequences.

A repeating element of DNA (1.053 kb) isolated from *B. pertussis* was sequenced revealing properties of an insertion sequence (IS) and was designated IS481 (McLafferty *et al.*, 1988). It was suggested the sequence could code for a transposase. A repeat DNA sequence of the same size was also shown to occur in at least 20 copies in *B. pertussis* (McPheat and McNally, 1987a). Sequence analysis of two of the twenty copies revealed that they were very closely related to the insertion sequence element IS481 and were subsequently labelled IS481v1 and IS481v2 (McPheat *et al.*, 1989a). Repeat sequences of 0.53 kb have also been identified and, although smaller than IS481, show considerable homology. They are thought to be present in 70-80 copies accounting for almost 1% of the bacterial genome (Park *et al.*, 1988). Another *B. pertussis* repeat DNA fragment (1.046 kb) has been cloned and sequenced revealing a close relatedness to IS481. It is believed to occur in 50-100 copies per cell with many of the copies arranged in tandem on the chromosome. These tandemly repeated sequence can be recognised by the distinct 1 kb fragment in ethidium bromide gels of *Cla*I digested *B. pertussis* chromosomal DNA. Though all these repeat sequences show considerable homology, small differences e.g. 12 bases difference between IS481 and the 1.046 kb repeat sequence, show that the sequences are not absolutely conserved.

The repeats IS481 (McLafferty *et al.*, 1988), 0.53 kb (Park *et al.*, 1988) and

1.046 kb (Glare *et al.*, 1990) are in unknown locations in the genome. However, a repeated DNA sequence has been located 210 bp upstream of the start codon of the porin structural gene in *B. pertussis* (Li *et al.*, 1991b). The *B. pertussis* repeated DNA sequences IS481v1 and IS481v2 are located close to at least two virulence genes. IS481v1 is located 3 kb upstream of the adenylate cyclase locus and IS481v2 is located immediately adjacent to the C-terminal end of the agglutinin 2 fimbrial subunit gene (McPheat *et al.*, 1989b).

IS481 and 0.53 kb repeats were not detected in *B. parapertussis* or *B. bronchiseptica* DNA (McLafferty *et al.*, 1988; Park *et al.*, 1988). IS481v1 and IS481v2 repeats were found to be absent in *B. parapertussis*, *B. bronchiseptica* (McPheat and McNally, 1987a) and in *B. avium* (McPheat and McNally, 1987b), although longer exposure revealed a homologous single copy band in *Bam*HI-digested *B. parapertussis* DNA (1.45 kb) and two bands in *Bam*HI-digested *B. bronchiseptica* DNA (1.45 kb, 10.1 kb) (McPheat and McNally, 1987a). Glare *et al.*, (1990) found that after prolonged autoradiographic exposure (4 days) one or two bands were detected in *B. bronchiseptica* DNA but none in *B. parapertussis* DNA. Analysis of repetitive DNA in *B. parapertussis* revealed an insertion sequence element, designated IS1001 (van der Zee *et al.*, 1993). The largest open reading frame showed homology with a putative transposase, designated TnpA, encoded by an insertion sequence element IS1096 found in *Mycobacterium smegmatis*. The insertion sequence was found in about 20 copies in all of the *B. parapertussis* strains analysed. An insertion sequence similar or identical to IS1001 was found in about five copies in *B. bronchiseptica* strains isolated from pigs and a rabbit. Neither *B. pertussis* nor *B. bronchiseptica* strains isolated from humans and dogs contained an IS1001-like element. Thus, repeated DNA segments, now recognised as insertion sequences, are found throughout the *Bordetella* genome.

McLafferty *et al.* (1988) identified a different repeat sequence adjacent to one copy of the repetitive DNA sequence, IS481. This was present in four copies

in *B. pertussis* and nine copies in *B. parapertussis*, suggesting that more than one kind of repetitive DNA sequence is present in the genomes of *Bordetella* (McLafferty *et al.*, 1988). Alsheikhly and Lofdahl (1989) also identified a repeat sequence, different from IS481, within the genome of *B. pertussis*. This consisted of an inverted repeat sequence surrounding a 200 bp central segment. It was present in more than one copy in *B. parapertussis* and also in *B. bronchiseptica*. They suggested that this IS-like element exists as an integral entity in just a few locations within the genome, but that the inverted repeat sequence is present in multiple copies. They also suggested that low or no toxin production by bordetellae may not only be due to point mutations, as suggested by Arico *et al.* (1987) but may be also due to DNA rearrangements involving repeat sequences. It was suggested that a potential binding site for the *bvg* gene-associated products was a set of tandem repeats upstream of the PT operon (Locht and Keith, 1986). Later, Gross and Rappuoli (1988) identified the regulatory region involved in the transcription of the PT operon as a 170 bp sequence upstream of the start site of transcription. A 21 bp repeated sequence in this region was proposed as the binding site for the trans-activating factor(s).

McPheat and McNally (1987a) suggested that DNA rearrangements at the sites of repeat DNA sequences might be involved in the regulation of virulence factors in *Bordetella*. Analysis of *B. pertussis* chromosomal DNA isolated from AGG2 serotype variants and from avirulent phase variants revealed no rearrangements around either of these two insertion sequences (McPheat *et al.*, 1989b), although, DNA rearrangements had been observed at other uncharacterised loci for phase variants of *B. pertussis* strain 18323 (McPheat *et al.*, 1987b). Repetitive sequences have been found to be associated with a number of virulence associated traits including the genes for *E. coli* heat-labile toxin (Yamamoto and Yokota, 1981) and haemolysin (Hacker *et al.*, 1983). The cholera toxin operon is flanked by tandem duplicated repeated DNA sequences (Goldberg and Mekalanos, 1986) and the *E. coli* heat-stable toxin by

inverted repeats of insertion sequence *IS1* which have been shown to transpose (So *et al.*, 1979).

Insertion sequences are a class of mobile genetic elements and one of several types of repeated DNA sequences. They are discrete units of DNA capable of inserting themselves at multiple sites in the bacterial genome (Iida *et al.*, 1983). They are classified as insertion sequences by several criteria: i) perfect or near perfect terminal inverted repeats of 10-40 bp; ii) two or three ORF's encoding transposition activity; iii) short duplication of target DNA at their insertion sites resulting in their being flanked by short direct repeats (2-12 bp) formed by the insertion of the IS element at its target site, the length but not sequence of the direct repeats being characteristic of each IS element; iv) they appear to encode only proteins that are involved in their own transposition, in contrast to transposons which carry drug and antibiotic resistance determinants. No significant DNA sequence homologies have been observed among different IS elements nor do their proteins bear any obvious amino acid homologies. The difference in the terminal inverted repeats of IS elements is thought to be specific for their transposition, where the inverted repeats act as the recognition sequences for the transposition enzymes, mutations within the sequences greatly affecting the mobility of the unit. It has been suggested that IS elements insert by creating a staggered cleavage in the target DNA at the insertion site. The short target DNA duplications would then reform by repair of the staggered ends for ligation with the IS element. If blunt rather than staggered cleavage were produced, there would be no duplications (Iida *et al.*, 1983).

Alternative effects of the IS family have been seen in prokaryotic gene expression by insertional inactivation or transposition of regions flanked by copies of the IS. IS elements appear to have been retained by bacteria where they appear to be important in mediating chromosomal rearrangements such as deletions, inversions and duplications and by altering the expression of adjacent genes. Such rearrangements may help organisms to adapt to new environmental

conditions (Arber, 1983). If IS elements are involved in evolutionary processes, it has been suggested the large copy number of the IS481 element in the most evolutionary advanced species of *Bordetella* might be important (McLafferty *et al.*, 1988). There may be an important functional role for *B. pertussis* in aiding its adaptation to new environmental conditions by controlling phase variation and virulence determinants. There is at present, however, no direct evidence for this.

1.8.3. Invertible DNA.

The reversibility of virulence factor expression might be accounted for by the *bvg* locus or its products being subject to regulation. Foxall *et al.* (1990) proposed that expression might be controlled by an invertible region of DNA in *Bordetella* affecting *bvg* expression. The homology was shown between *Bordetella* DNA and a *hin* region DNA probe by DNA hybridisation. These results were corroborated by MacGregor (1990). The DNA invertase system in other bacteria will now be reviewed.

1.9. DNA INVERSION IN OTHER BACTERIA.

1.9.1. The DNA invertase, Din, family.

Several invertible DNA mechanisms are very closely related, forming a single family of invertible DNA systems known as din (DNA inversion). The DNA invertases mediate "genetic switches" (Plasterk and van de Putte, 1984; Glasgow *et al.*, 1989) where inversion of a DNA segment, flanked by inversely repeated recombination sites, can switch expression between two alternative sets of genes. In the reactions catalysed by the DNA invertases Hin (*S. typhimurium*), Gin (phage Mu) and Cin (phage P1), the factor for inversion stimulation, FIS, stimulates recombination by binding to an enhancer sequence. Within the enhancer, two FIS dimers bind to two 15 bp consensus sequences and induce bending of the DNA. Current models propose that the enhancer-FIS complex organises a specific synapse, either through direct interaction with the invertase

protein, or by modelling the substrate into a configuration suitable for recombination (Finkel and Johnson, 1992). Interestingly, cellular concentrations of FIS vary tremendously under different growth conditions. This might have important regulatory implications for the physiological role of FIS in these different reactions. One feature of FIS-binding sites is a propensity for A/T rich regions flanking the core, consisting of runs of 3-6 As or Ts, flanking the core. The *hin* and *cin* enhancers contain two FIS-binding sites whereas the *gin* enhancer contains three, although only two are required to stimulate inversion.

The *Hin* enhancer consists of a 63 bp DNA sequence containing two binding sites for FIS. During the inversion reaction, the recombinational enhancer becomes physically associated with the two recombinational sites. This nucleoprotein complex, called an invertasome, has been visualised by electron microscopy. The assembly of the invertasome is believed to initiate with the joining of recombinase dimers bound at the two recombination sites followed by the incorporation of the Fis-bound enhancer DNA segment to form a branched structure on supercoiled DNA (Finkel and Johnson, 1992). It has been proposed that a conformational change in the recombinase, induced upon assembly of the enhancer into the recombinational complex, may weaken the recombinational contacts allowing subunit, hence DNA strand, exchange (Johnson, 1991). Fig. 1.5. shows a schematic representation of the molecular process of the *Hin*-mediated inversion reaction. FIS was originally discovered because of its critical role in promoting site-specific recombination in bacteriophages. Recent discoveries have shown a role for this protein in the transcription of operons whose products are central to protein synthesis with the need for rapidly changing expression. Finkel and Johnson (1992) have speculated that it may have an important role in mediating rapid responses in cell growth as a function of environmental fluctuations.

Within the *Din* family, the *hin* invertase system of *S. typhimurium* is involved in flagellar phase variation. The *gin* system of bacteriophage Mu and

Fig. 1.5. Schematic representation of the molecular process of the Hin-mediated DNA inversion reaction.

The H region in the chromosome of *S. typhimurium* is shown. Hin binds to *hix* recombination sites as a dimer while Fis binds independently to each domain of the recombinational enhancer as a dimer (**Binding**).

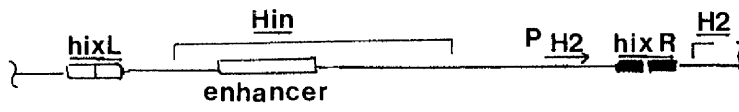
Interaction between Hin dimers bound to two *hix* sites brings two recombination sites close together (**Pairing of hix sites**).

A productive synaptic complex is formed at the enhancer between paired *hix* and Hin complex and Fis (**Invertasome formation**).

Hin cleaves in the centre of each *hix* site followed by strand exchange and religation (**Cleavage and strand exchange**).

The resulting recombination product has inverted the genetic information between the *hix* sites. P_{H2} , promoter for the *H2* gene. (Redrawn from Lim and Simon, 1992).

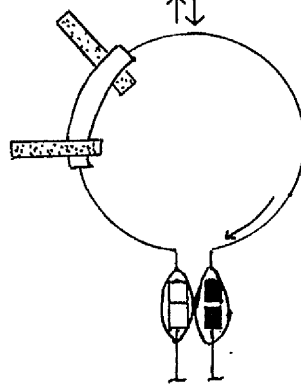
H2 on;



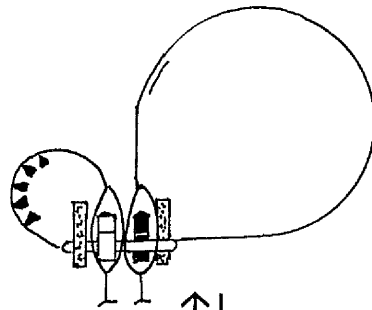
↑↓ Hin ○ Binding
Fis ▤



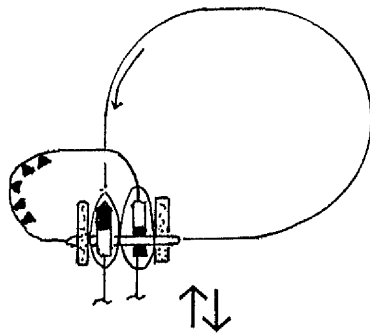
Pairing of
hix sites



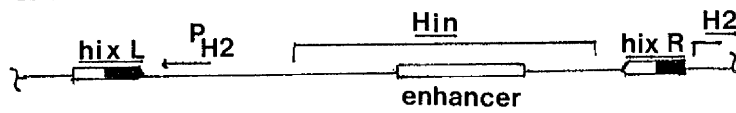
↑↓ HU ▲ Invertasome
formation



Cleavage and
strand exchange



H2 off;



the *cin* system of bacteriophage P1 are both involved in determining the host range of the phages via the expression of different tail fibre proteins, while the *pin* system of *E. coli* has no known function. Sequence homology to these has also been shown with *min*, a DNA segment on the *E. coli* plasmid p15B, and *pinB* and *pinD* of *Shigella*. *Moraxella bovis* exhibits pilin variation, and homology to the invertase inverted repeats and enhancer region have been detected although there was no significant sequence similarity to the invertases. The Bin protein from *Staphylococcus aureus* also shows homology to the Din proteins and was originally thought to be an invertase by sequence analysis, although no such function has been established.

1.9.1.a. Hin DNA inversion in *S. typhimurium*.

S. typhimurium undergoes variation between two serospecific flagellar filaments, H1 or H2, and can switch reversibly between the two states effecting a phase change and antigenic variation simultaneously. New symbols for the flagellar structural genes *H1* and *H2* are *fliC* and *fliB* respectively as suggested by Iino *et al.* (1988) though both old and new terms are still in use. The older symbols of *H1* and *H2* are used here. The rate of this phase change is of the order of 10^{-3} - 10^{-4} per cell division. The molecular mechanism for the switch involves the reversible inversion of a 995 bp sequence of the *S. typhimurium* chromosome that is bounded by 14 bp inverted repeat sequences. Inversion is mediated by the *hin* gene product which is encoded within the segment and is expressed in either orientation. The *hin* gene product catalyses site-specific recombination between the inverted repeats at either end of the invertible segment and to cause inversion (Zieg *et al.*, 1977; Silverman *et al.*, 1979a; Simon *et al.*, 1980).

The invertible region carries a promoter (P) and in one orientation (phase H2) the promoter directs transcription of the *H2* gene, leading to synthesis of H2 flagellin, and of the *rh1* (*fliA*) gene which encodes a trans-acting

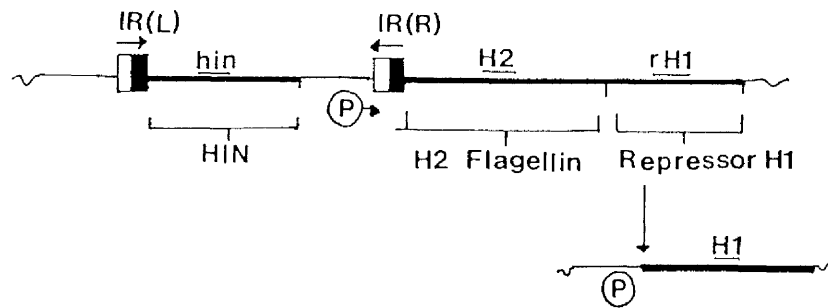
protein repressor that represses expression of the *H1* gene (Silverman *et al.*, 1979b). In the opposite orientation (phase H1) the promoter cannot read through either *H2* or *rh1*. Therefore *H2* flagellin is not made and the repression of *H1* expression is relieved. The alternative flagellin gene *H1* is located distantly from the *H2* and the invertible control region (Saunders, 1986) whose nucleotide sequence has been determined (Silverman *et al.*, 1981; Fig. 3.2.). The site-specific inversion also involves protein HU as well as *Hin* and FIS. HU is a non-specific DNA binding protein possibly enabling bending of the DNA (Johnson and Bruist, 1989). The enhancer and the DNA supercoiling rotate the DNA strands subsequent to double stranded cleavage by *hin* within both recombination sites. Ligation of recombinant configuration results in inversion of the intervening DNA (Johnson and Bruist, 1989). Fig. 1.6. shows a schematic representation of the genetic rearrangement controlling *S. typhimurium* flagellar phase transition.

The DNA sequences comprising the *H2* locus (invertible region and *H2* structural gene) have been compared with the Mu phage sequences and the sequences including and 5' to the *H1* and *hag* genes (Szekely and Simon, 1982). The homology shown suggested to Silverman and Simon (1983) that the *H2* region was a fusion containing sequences from two different sources. There was marked homology between *H2*, *H1*, and *hag* gene sequences, and this close similarity extended for approximately 100 bp from the N-terminus of these genes into the 5' noncoding region. At this point, there was an abrupt discontinuity where the *H2* locus sequences diverged sharply from the *H1* and *hag* sequences, which continued to resemble each other. Approximately 300 bp from the N-terminus of the *H2* gene, considerable homology with Mu sequences was detected. This region of homology with Mu (shown in Fig. 1.7.) extended for 700 bp, the IR(L)-*hin* region of *Salmonella* having extensive homology with the IR(R)-*gin* region of Mu suggesting it was probably derived from a *gin*-like sequence. A point approximately 100 bp 5' from the start of the *H2* gene

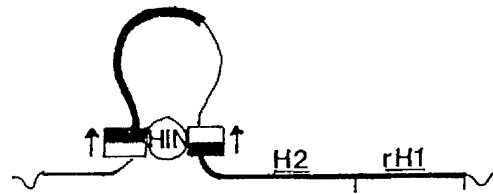
Fig. 1.6. Genetic rearrangement controlling *S. typhimurium* flagellar phase transition.

Inversion of a region of DNA of approximately 1000 bp adjacent to the *H2* operon alternatively couples and uncouples a promoter element. When the promoter is coupled to the operon *H2*, flagellin is synthesized as well as the product of the *rhI* gene that represses *H1* expression (i; *H2* on, *H1* off). When the promoter is uncoupled from the *H2* operon, no *rhI* gene product is synthesized, and the *H1* gene is expressed (iii; *H2* off, *H1* on). IR(L) and IR(R) are the cis-acting sites in inverted repeat configurations in which a reciprocal recombination event resulting in inversion takes place. The *hin* gene product is encoded by a sequence within the inversion region and is required for inversion. The fate of DNA sequences rearranged by inversion can be followed by referring to the filled areas of the IR boxes. Note that IR(L) and IR(R) are defined for the *H2* (on) position. (Redrawn from Silverman and Simon, 1983).

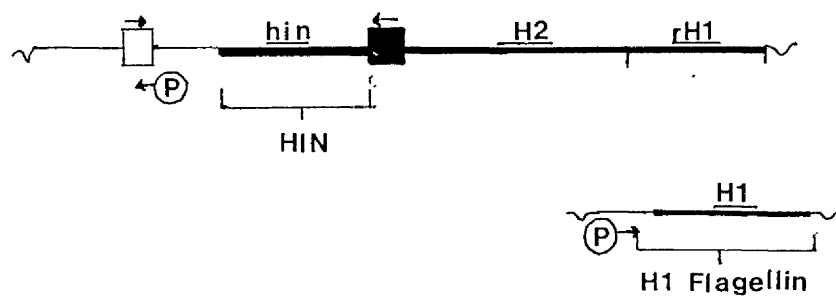
(i)



(ii)



(iii)



marked a boundary between transposon-like and *HI*-like sequences. Thus, the IR(R) sequence in the phase variation system was embedded in and probably was derived from *HI*-like sequences. In fact, the sequence -ATCAA- which was part of the 14-bp IR(R) recognition sequence was conserved in the same relative position in *HI* and *hag* (Silverman and Simon, 1983).

These sequence relationships suggested to Silverman and Simon (1983) a process for the evolution of the phase variation system. Hypothetical steps in the evolutionary process have been outlined in Fig. 1.8. The IR(L)-*hin* recombinase could be derived from a transposon-like unit that inserted adjacent to an ancestral *HI* gene. A low frequency recombinational event at a site adjacent to that of the flagellin gene could capture the promoter sequence of that gene. Subsequent repair or recombinational processes similar to those of gene conversion could lead to homogenisation of the sequence immediately adjacent to the recombination site, with the resultant generation of a functional IR(L)-IR(R) site pair. The consequences of such a promoter capture would be on-off switching of flagellin synthesis mediated by the recombinase function. The phase variation system could have developed as a result of a number of steps, including the insertion of a mobile element adjacent to an ancestral *HI* gene and the capture of the *HI* promoter by a recombinational function that was part of the mobile element.

1.9.1.b. *Gin* DNA inversion in bacteriophage Mu.

In bacteriophage Mu, a switch in host range is caused by site-specific inversion of the 3 kb G segment (van de Putte *et al.*, 1980). Inversion provides the means to alternate the expression of two sets of genes that are involved in tail fibre biosynthesis (Grundy and Howe, 1984). The recombination sites are 34bp long and are arranged as inverted repeats (Plasterk *et al.*, 1983). The product of the gene, *gin* (G inversion) which lies outside the G region, is responsible for the site specific recombination that inverts the G region.

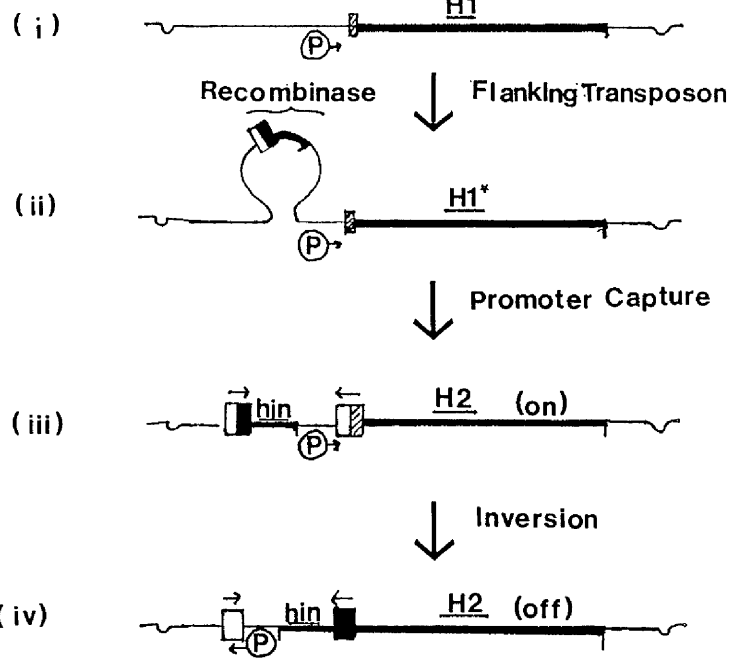
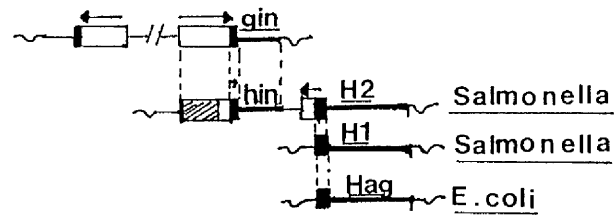
Fig. 1.7. Sequence relationships. At least two different sources contributed to the DNA sequence of the *H2* region.

The *H2* structural gene and a region approximately 100 bp N-terminal to the start of *H2* has a high degree of DNA homology with the *H1* region and with the *hag* region in *E.coli*. This region extends inside the inversion region and includes the *H2* gene promoter. Past this boundary, about 100 bp left of IR(R), the DNA sequence resembles that of the IR(R)-*gin* region of Mu. IR boxes are not drawn to genetic scale. (Redrawn from Silverman and Simon, 1983.)

Fig. 1.8. Evolutionary considerations to account for the origin of sequences in the *H2* gene switch.

A mobile genetic element similar to the TnA class of transposons inserted adjacent to an ancestral *H1* gene (i, ii). Genetic alteration of a site between the $H1^*$ promoter and the $H1^*$ gene led to a functional recombination site similar to but in inverted configuration to the recombination site carried by the recombinase (iii). The recombinase function (*hin*) acting on the pairs of recombination sites inverts the region containing the *H2* gene promoter (iv). The recombinase that originally was an integral part of a transposon now functions to regulate an unrelated gene system. (Redrawn from Silverman and Simon, 1983).

Mu G region



Transcription and translation originates from a region that lies outside G through a protein-coding region called Sc (constant) and can lead to the production of alternative pairs of proteins. In one orientation a protein containing Sc fused to Sv (variable), and a second protein called U are made. These confer on Mu the ability to infect *E. coli*. In the other orientation of G, proteins Sc-S' and U' are made which allow the phage to propagate on *Citrobacter freundii* (Saunders, 1986). Fig. 1.9. shows a schematic representation of the genetic rearrangement in bacteriophage Mu.

1.9.1.c. Cin DNA inversion in bacteriophage P1.

Phage P1 has a similar invertible segment and site specific recombination mechanism that interchange with that of Mu. Phage P1 contains an invertible DNA segment of 4200 bp flanked by 640 bp inverted repeats (Hiestand-Nauer and Iida, 1983). The enzyme for P1 inversion is encoded by the *cin* gene and also controls the host range of the phage. The proteins encoded by *hin*, *gin*, and *cin* share 60-70% amino acid sequence identity and have been shown to function on each other's DNA substrate. Differences in structure arise due to *gin* and *cin* DNA invertase genes being adjacent to the invertible DNA rather than being part of the structure as with *hin* (Johnson and Bruist, 1989).

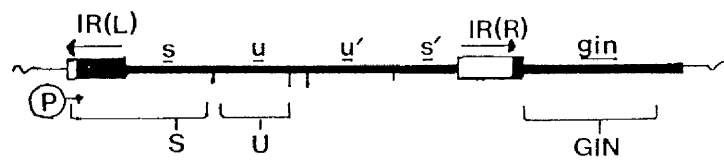
1.9.1.d. Pin DNA inversion in *E. coli*.

E. coli harbours a site-specific inversion system of the defective phage ϕ 14 (Greener and Hill, 1980). *Pin*, the invertase gene, acts on the adjacent P region of invertible DNA. *Pin* has functional homology to the invertases of Mu and P1 and suppresses *hin* mutations of *Salmonella* (Kutsukake *et al.*, 1985). As yet, no function has been ascribed to the invertible DNA. However it is known that the inversion determines the differential expression of at least one protein (Plasterk and van de Putte, 1984), although alteration in protein expression has not been correlated with any apparent change in the phenotype of *E. coli*.

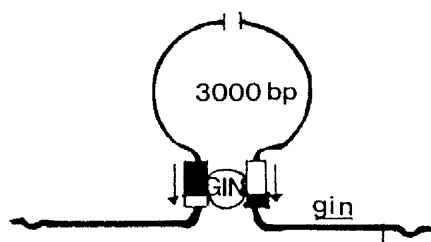
Fig. 1.9. Mu host range variation determined by a genetic rearrangement.

The host range of the phage is determined by the presence of pairs of gene products in the infectious particle. If gene products S and U are present, the phage will infect *Escherichia coli* (i); if gene products S' and U' are present, the phage will infect *Citrobacter* and other enterobacteria (iii). The alteration in gene product synthesis is controlled by the inversion of a 3000-bp region that encodes an s, u operon and an s', u' operon in inverted configuration (ii). The promoter and a small part of the coding region lie outside the inversion region. In contrast to *S. typhimurium* phase variation, alteration of expression is by reversible coupling of different coding regions with a static promoter. As in Fig. 1.6, the cis-acting sites for inversion are marked IR(L) AND IR(R). These regions of inverted homology are longer than those of *Salmonella* phase variation (34 bp versus 14 bp). The *gin* gene, located outside the inversion region, encodes a protein required for inversion. (Redrawn from Silverman and Simon, 1983).

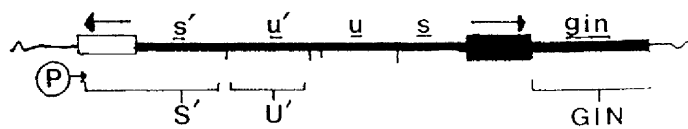
(i)



(ii)



(iii)



1.9.2. DNA inversions proposed to be part of the Din family.

1.9.2.a. Min DNA inversion in *E. coli* 15 T⁻.

The Min DNA inversion enzyme of plasmid p15B of *E. coli* 15T⁻ has been examined by Iida *et al.* (1990). Plasmid p15B is a bacteriophage P1-related resident of *E. coli* 15T⁻. Both *E. coli* and P1 genomes contain a segment in which DNA inversion can occur, although these segments are not identical. Cloning of the Min DNA segment into a plasmid resulted in multiple DNA inversions of the 3.5 kb segment on the recombinant plasmid. The DNA inversion was therefore called Min. The *min* gene can complement the P1 *cin* recombinase gene. DNA sequence analysis of the *min* gene shows that Min is most closely related to the Pin recombinase of the e14 defective viral element on the *E. coli* K12 chromosome. The *min* gene contains a recombinational enhancer element which stimulates site-specific DNA inversion 300-fold.

1.9.2.b. Din DNA inversion in *Shigella*.

The invertible B segment DNA and *pinB* recombinase gene cloned from *Shigella boydii* are highly homologous to the phage Mu G invertible region and *gin* in size, organisation and nucleotide sequence of open reading frames (Tominga *et al.*, 1991). It is not known whether the invertible DNA segment B and recombinase *pinB* gene structure are part of a defective prophage derived from Mu-like phage or whether it is a progenitor of a phage like Mu. If the invertible B segment DNA and recombinase *pinB* gene are part of a prophage, the products of the ORFs on the B segment and its flanking region would presumably be tail fibre proteins concerned with host recognition (Tominaga *et al.*, 1991).

1.9.2.c. DNA inversion in *Moraxella bovis*.

M. bovis EPP63 is able to produce two antigenically distinct pili named Q

and I (previously called beta and alpha). The phase variation between Q and I pilin gene expression is associated with the inversion of a 2.1 kb DNA fragment whose ends occur within the coding region of the expressed pilin gene (Marrs *et al.*, 1988). Analysis of the beta-pilin gene revealed a stretch of 26 bases at the amino-terminal portion with homology to the left inverted repeat of the *S. typhimurium* flagellar *hin* control region. In addition, Fulks *et al.* (1990) found 50% sequence homology to the recombinational enhancer of bacteriophage P1. Two ORFs representing potential genes were also identified. One was a strong candidate for an invertase, *piv*, but there was no significant sequence homology to the Din family of invertases (Marrs *et al.*, 1990).

1.9.2.d. Bin DNA inversion in *S. aureus*.

The nucleotide sequence of a staphylococcal plasmid gene "*bin*" was predicted to encode a protein homologous to proteins in the Din family (Rowland and Dyke, 1988). The staphylococcal invertible region (*inv*) was first characterised as a 2.2kb segment flanked by 650 bp inverted repeats. Although originally identified as an invertase, it was shown to be related to Tn3. Later work suggested that Bin is a homologue of Tn552 resolvase (a *S. aureus* beta-lactamase-containing transposon) rather than an invertase (Rowland and Dyke, 1989). This may therefore represent a link between invertases and the resolvases, where *bin* determines a resolvase which can occasionally mediate DNA inversion (Iida *et al.*, 1990).

1.9.3. Transposons.

The Din family share sequence homology and mechanistic features with the resolvase family of recombinases, for example, TnpR of Tn3-class transposons (Glasgow *et al.*, 1989). To enable recombination to occur, the transposons of the Tn3 family encode two specialised recombination systems. The first consists of the transposase protein, TnpA, which interacts with the two

ends of the transposon, to insert the entire molecule into the target. The second transposon recombination system, TnpR, and the recombination site, resolvase or res, resolves the recombination product into the two products. This second, site-specific recombination protein, TnpR shows amino acid sequence homology to the invertases previously mentioned. The structure of various transposons are shown in Fig. 1.10.

There is little similarity between the Tn3-type transposases and the other elements and no homology has been found between transposases and recombinases/topoisomerases. Most workers have suggested, on the basis of the common transposition mechanism, that the transposase catalytic activity resides in the C-terminal region of the transposase. A phylogenetic tree for the relationships among the resolvases/invertases is shown in Fig. 1.11. No formal derivation of a phylogeny for repeats or transposases has been reported. However, if the phylogenies are similar, it suggests that the transposases and resolvases have largely co-evolved and have not been subject to frequent reassortment (Sherratt, 1989).

1.10. AIMS OF RESEARCH.

A previous study had revealed homology between the *hin* region of *S. typhimurium* and a *B. pertussis* chromosomal DNA fragment. This study was designed to determine the identity of this homologous *B. pertussis* fragment and to determine whether the homology was due to an invertase sequence and, if so, what its function was in the *B. pertussis* genome. This study was to include the other three *Bordetella* species in addition to *B. pertussis*. All four species chromosomal DNA were to be examined for evidence of differential methylation related to phase variation which had also been suggested by a previous study.

Fig. 1.10. Three classes of bacterial transposons.

(A). ISs and composite transposons. ISs range in size from about 750 bp to 1600 bp. They generally have a single transposase gene (*tnp*). Composite transposons consist of a DNA segment (often with a phenotypic determinant such as a drug-resistance gene) flanked by two copies of an IS; the IS copies may be in the same or inverted orientations.

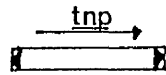
(B). Tn3-family transposons. *tnpA* and *tnpR* are the genes for transposase and the cointegrate resolvase respectively, *res* is the site of cointegrate resolution. Two subgroups have been distinguished and are exemplified by (i) Tn3 and (ii) Tn501. In these subgroups the DNA segment that contains *tnpR* and its *res* site has adopted opposite orientations. The *tnpA*-(*res-tnpR*) segment is about 3800 bp in size. The remainder of the transposon may carry additional genetic markers (e.g. ampicillin resistance in Tn3, mercury resistance in Tn501).

(C). Mu. The whole genome is about 38 kb. The A and B genes are necessary for efficient transposition and replication. Near the right end lies a 3 kb invertible DNA segment (the G segment) and the *gin* gene; *gin* is related to the *tnpR* genes of Tn3 and Tn501 and encodes a site -specific recombinase that inverts the G segment. (Redrawn from Grindley and Reed, 1985).

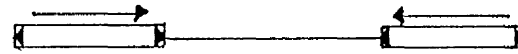
Fig. 1.11. Phylogenetic tree derived by analysis of the primary structures of the site-specific recombinases or resolvases.

Res is carried on the *Clostridium perfringens* plasmid pIP404. The RES protein is highly homologous to the resolvases of transposons as well as enzymes involved in site-specific DNA inversion. Phylogenetic analysis indicates a common descent for ten site-specific recombinases and showed the RES protein to be closest to the resolvase encoded by Tn917 from *Streptococcus faecalis*. (Redrawn from Garnier *et al.*, 1987)

A
(i) IS

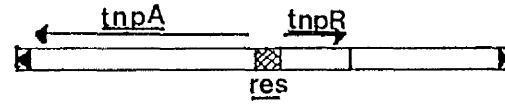


(ii) Composite Tn

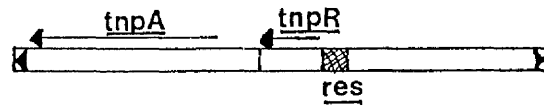


B

(i) Tn₃

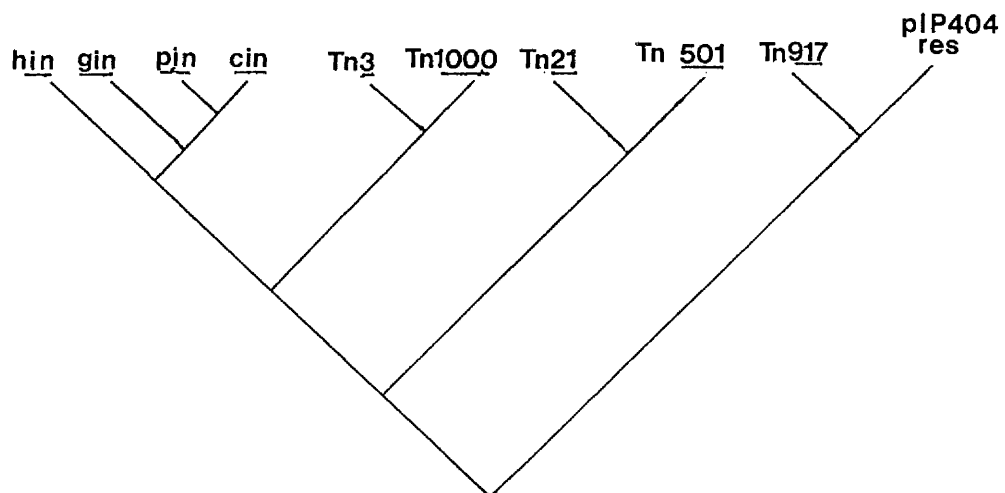


(ii) Tn 501



C

Mu



CHAPTER 2

MATERIALS AND METHODS.

2.1 SOURCES OF MATERIALS AND CHEMICALS.

Unless stated otherwise, all chemicals were obtained from BDH Chemicals Ltd., Poole, U.K. and were of analytical reagent grade whenever possible. Distilled water (DW) was double distilled. Sterile distilled water (SDW) was from West Midlands Regional Sterile Supply Unit, Wolverhampton.

Amersham International, Bucks, U.K. :

(α -³⁵S) dATP, 1000 μ Ci/mM; (α -³²P) dATP (aqueous), 400 μ Ci/mM; (γ -³²P) dATP (aqueous) 3000 μ Ci/mM. Multiprime DNA labelling system.

Bio-Rad Laboratories, Watford, Herts., U.K. :

Acrylamide, methylene-bis acrylamide, "Zeta probe" nylon membrane.

Difco Laboratories, East Molesey, Surrey, U.K. :

Agar No.3, casamino acids, tryptone, yeast extract.

Gibco-BRL, Paisley, U.K. :

Agarose, ammonium persulphate, Bordet Gengou agar, caesium chloride, calf intestinal phosphatase (CIP), dialysis tubing, formamide, isopropylthio- β -D-galactoside (IPTG), lambda DNA, phenol, T₄ DNA ligase and buffer, N,N,N',N'-tetramethylethylene-diamine (TEMED), urea (ultra-pure grade), DNA kilobase ladder.

May and Baker Ltd, Dagenham, UK. :

Diethyl ether.

Oxoid, Basingstoke, Hants. :

Cephalexin, nutrient agar, nutrient broth.

Sigma Chemical Company (UK) Ltd., Poole, Dorset, U.K. :

Ampicillin (sodium salt), ascorbic acid, bovine serum albumin (BSA pentax fraction V), 5-bromo-4-chloro-3-indolyl- β -D-galactoside (BCIG/X-gal), bromophenol blue, Brij 58, DL-dithiothreitol (DTT), EDTA, erythromycin, ethidium bromide, Ficoll, glucose, glutathione (reduced), glycerol, isoamyl alcohol, light mineral oil, low gelling temperature agarose (Type XI), lysozyme, L-proline, monosodium glutamate, nicotinic acid, polyethylene glycol (PEG Mr. 6000), polyvinylpyrrolidone, proteinase K, salmon sperm DNA, tetracycline, tris base, thiamine, xylene cyanol FF.

Stratech, Scientific Ltd, U.K. :

GeneClean II kit.

Tissue Culture Services Ltd, Buckingham, UK. :

Defibrinated horse blood.

2.2 MEDIA PREPARATION.

All media and sterile equipment used in this study were sterilized for 15 min at 121°C (15 lbs/in²) before use.

Bordetella growth.

Bordetella strains were grown in 200 ml volumes of cyclodextrin liquid medium (Imaizumi *et al.*, 1983) consisting of three parts:- basal medium (with cyclodextrin), supplement and casamino acids.

Basal medium.

For one litre of medium:-

Monosodium glutamate (L-glutamic acid)	10.70 g
L-proline	0.24 g
NaCl *	2.50 g
KH ₂ PO ₄	0.50 g
KCl	0.20 g
MgCl ₂ .6H ₂ O	0.10 g
CaCl ₂ .2H ₂ O	0.02 g
Tris base (Trizma)	1.5 g
Heptakis (2,6-0-dimethyl) beta-cyclodextrin **	1.00 g

Distilled water was added and the pH adjusted to 7.4 with concentrated HCl, to give a final volume of 1 litre. The medium was then autoclaved and stored at room temperature. The ingredients for 5 litres were usually made in a total volume of 1 litre and stored as a 5 x stock.

* To grow *Bordetella* in the C-mode, the NaCl was replaced with MgSO₄, 2.45 g/l.

** The cyclodextrin was kindly supplied by Teijin Ltd, Tokyo, Japan.

Basal medium growth supplement.

L-cysteine	0.4 g
FeSO ₄ ·7H ₂ O	0.1 g
Niacin (nicotinic acid)	0.04 g
Glutathione (reduced)	1.5 g
Ascorbic acid	4.0 g

Distilled water was added to a total volume of 100 ml and the medium filter-sterilized using 0.22 µm Millipore filters. The supplement was divided into 10ml aliquots and stored at -20 °C. The supplement was used at 10 ml per 1 litre basal medium.

Casamino acids.

Casamino acids, stock 25% (w/v) solution in distilled water was autoclaved separately and stored at room temperature. This solution was added to the medium to give 1% (v/v).

Bordet Gengou agar.

Bordet Gengou agar, BGA, 36 g, and glycerol, 1% (v/v, final) were added to distilled water to give a final volume of 850 ml. This was boiled to dissolve the solids and then autoclaved. The agar was allowed to cool to 45-50 °C before adding 150 ml of sterile warm (37°C) fresh defibrinated horse blood (Tissue Culture Services Ltd, Buckingham, U.K.). Overlay plates had a bottom agar of BGA without blood (12 ml) and a top layer BGA with blood (12 ml). This aided the visualisation of haemolysis.

E. coli growth.

L broth medium (pH 7.4).

Tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	10 g/l

The pH was adjusted to pH (7.4) with 5M NaOH, 15 g/l Difco agar was added to solidify the medium when appropriate.

Nutrient agar.

Nutrient agar, NA (Oxoid) prepared using 28 g/l in distilled water.

Nutrient broth (No. 2 Oxoid) prepared as 14 g/l in distilled water.

2 x YT (pH 7.4).

Tryptone	16 g/l
Yeast Extract	10 g/l
NaCl	5 g/l

The pH was adjusted to 7.4 with 5M NaOH.

15 g/l Difco agar was added to solidify the medium when appropriate.

Defined minimal medium, M9 (10x stock).

Na ₂ HPO ₄	60.0 g/l
KH ₂ PO ₄	30.0 g/l
NaCl	5.0 g/l
NH ₄ Cl	10.0 g/l

The pH was adjusted to 7.4 with 10 M NaOH prior to autoclaving. An appropriate volume of the above 10x stock was added to molten (50 °C) 2% (w/v) Difco agar in distilled water prior to the addition of the following constituents:

	per 200 ml
1 M MgSO ₄ (autoclaved separately)	2.0 ml
0.1 M CaCl ₂ " "	0.1 ml
20% glucose " "	10.0 ml
Thiamine (100 µg/ml)	2.0 ml

M13 phage.

H-top agar (pH 7.4).

NaCl	8 g/l
Agar(Difco)	8 g/l

The pH was adjusted to 7.4 with 1M HCl.

2.3. BUFFERS AND SOLUTIONS FOR DNA TECHNIQUES

Information not included elsewhere:

TE (1x) (pH 8.0).

Tris-HCl	10 mM
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EDTA	1 mM
------	------

The pH was adjusted to 8.0 with 5M NaOH.

Preparation of chromosomal DNA.

Saline EDTA (pH 8.0).

NaCl	0.15 M
------	--------

EDTA	0.1 M
------	-------

The pH was adjusted to pH 8.0 with NaOH.

TE-saturated phenol.

Phenol (Gibco-BRL) was stored at -20°C and melted by placing the bottle in a 60°C water bath. To the phenol an equal volume of 100 x TE was added and mixed, the mixture was centrifuged, and TE phase removed. Phenol: chloroform: isoamylalcohol (25:24:1) was made as needed.

Preparation of plasmid DNA.

T.E.S. (pH 8.0).

Tris-HCl	10 mM
----------	-------

EDTA	1 mM
------	------

NaCl	100 mM
------	--------

The pH was adjusted with 5M NaOH.

Dialysis tubing (washed).

Dialysis tubing (Gibco-BRL) was supplied in 25% (v/v) ethanol, 1 mM EDTA, 1 mM sodium azide and required only a rinse in distilled water prior to use.

Electrophoresis.**TBE buffer (10x) (pH 8.3).**

Tris-base	0.9 M
Boric acid	0.9 M
EDTA	0.03 M

The pH was adjusted to 8.3 with NaOH.

Agarose gel loading dye.

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Ficoll	15% (w/v)

Ethidium bromide

This was made up as 10 mg/ml stock in water. The container was wrapped in aluminium foil and stored at room temperature.

Southern blotting.**SSC buffer (20x) (pH 7.0).**

NaCl	3.0 M
Na ₃ citrate.2H ₂ O	0.3 M

Radiolabelling of oligonucleotide probes.

Kinase buffer (KB) (10x) (pH 7.4).

Tris-HCl	500 mM
MgCl ₂	100 mM
DTT	50 mM

The pH was adjusted to 7.4 with 1M HCl and the solution stored at -20°C.

DNA Hybridisation.

Denhardt's reagent (50x).

	g l ⁻¹
Polyvinylpyrrolidone	10.0
BSA (pentax fraction V)	10.0
Ficoll (Type 400)	10.0

This was filter sterilized or made in sterile distilled water and stored at -20 °C.

Polyacrylamide gels (for DNA sequencing).

40% Acrylamide stock.

	g l ⁻¹
Acrylamide	380
Bis-acrylamide	20

This was made up to one litre with distilled water, mixed with 20g amberlite MB-1 resin stirred for 30 min and filtered.

0.5 x TBE gel mix (A).

10 x TBE buffer	50 ml
40% acrylamide stock	150 ml
Urea (ultra-pure)	460 g

This was made up to 1 litre with distilled water.

5.0 x TBE gel mix (B).

10 x TBE buffer	500 ml
40% acrylamide stock	150 ml
Urea (ultra-pure)	460 g
Bromophenol blue	0.05 g

This was made up to 1 litre with distilled water.

2.4. BACTERIAL STRAINS AND RECOMBINANT DNA VECTORS.

2.4.1. *Bordetella* strains.

Various strains of the four *Bordetella* species were examined, including serotype variants of *B. pertussis* and phase variants of *B. pertussis* (I and IV) and *B. bronchiseptica* (I and III). For *B. parapertussis* and *B. avium*, only the virulent strains were available. A description of the source and serotype of the *Bordetella* strains is given in Table 2.1. *B. pertussis* Wellcome 28 is a vaccine strain originally from P. Novotny (Wellcome Laboratories, Beckenham, Kent) . The strains, Arg 40 and Arg 41, are serotype variants of the strain Wellcome 28 (Robinson *et al.*, 1989b).

2.4.2. Other bacteria.

Other bacterial species used in this study and their sources are listed in Table 2.2.

2.4.3. Cosmid, plasmid and phage vectors.

These are listed in Table 2.3.

2.5. MEDIA AND GROWTH OF BACTERIA.

Details of media composition and preparation are given in Section 2.2. Short term storage of cultures was on agar plates at 4 °C, whereas long term storage was at -70°C, as broth cultures supplemented with 50% glycerol (v/v).

Bordetella strains were grown in a moist atmosphere on Bordet-Gengou Agar supplemented with 15% (v/v) defibrinated horse blood and cephalixin (40 µg/ml) for 96 h (*B. pertussis*) or 24 h (*B. parapertussis*, *B. bronchiseptica*, and *B. avium*). Cultures were routinely checked for purity by streaking on BGA plates without cephalixin and by Gram staining. Liquid cultures were grown in

Table 2.1. *Bordetella* strains and serotypes used in this study.

Strain/phase	Serotype	Source
<i>Bordetella pertussis</i> :		
Taberman	1,0,3	Professor G.T. Stewart ^a
Tohama	1,2,0	Dr A. Robinson ^b (*)
Wellcome 28 (W28)	1,2,3	..
Arg 40	1,2,0	..
Arg 41	1,0,3	..
364 IV	-	NCTC 10901
L84 I	1,2,0	NCTC 11089 (**)
L84 IV	-	NCTC 10902 (**)
D30042 I	1,2,3	NCTC 10911
D30042 IV	-	Dr J. Dolby ^c
134 I	1,0,3	..
134 IV	-	..
11615 IV	-	..
M2 I	1,0,3	Dr.N. Preston ^d
<i>Bordetella parapertussis</i> :		
5952	Type strain	NCTC 5952
10520		NCTC 10520
<i>Bordetella bronchiseptica</i> :		
452	Type strain	NCTC 452
AS1 I		Dr A.J. Lax ^e
AS1 III		..
276 I		..
276 III		..
FW5 I		..
FW5 III		..
<i>Bordetella avium</i>		
4148		Dr R. Rimler ^f .
4480		..

NCTC: National Culture Type Collection

^a Ruchill Hospital, Glasgow.

^b Biologics Division, PHLS CAMR.

^c Clinical Research Centre, Middlesex.

^d Manchester University.

^e Institute for Research on Animal Diseases, Newbury, Berks.

^f University of Ames, Iowa, USA.

(*) Sato and Arai (1972).

(**) Ackers and Dolby (1972).

Table 2.2. Other bacteria.

Strain	Source	Genotype and reason for use
<i>E. coli</i> DH1	A	<i>supE44, hsdR17, recA1, endA1, gyrA96, thi⁻1, relA1, B. pertussis</i> library including cosmid clone, p16.
<i>E. coli</i> JM83	A	F ⁻ <i>ara, Δ(lac-proAB) rpsL, [φ 80 (d lacZ ΔM15)], flagellate⁺, Gram⁻.</i>
<i>E. coli</i> TG1	B	<i>supE, hsdΔ5, thiΔ(lac-proAB) F' [traD36, proAB⁺, lacI^q lacZ ΔM15],</i> for phage replication.
<i>E. coli</i> GM242	C	<i>dam⁻.</i> For plasmid propogation in Dam ⁻ conditions.
<i>S. typhimurium</i> LT2 EMG36	A	<i>hin⁺, flagellate⁺, Gram⁻.</i>
<i>Klebsiella aerogenes</i>	D	<i>flagellate⁻, Gram⁻.</i>
<i>Bacillus stearothermophilus</i>	E	<i>flagellate⁺, Gram⁺.</i>
<i>Peptostreptococcus magnus</i> W1384	F	<i>flagellate⁻, Gram⁺.</i>
<i>Clostridium acetobutylicum</i>	B	<i>flagellate⁺, Gram⁺.</i>

(A): Dr J. Coote, Department of Microbiology, University of Glasgow.

(B): Dr N.P. Minton, (E): Dr R. Hutson, (F): Dr J. Murphy, Division of Biotechnology, PHLS, CAMR.

(C): Dr G. Wilkinson, (D): Mr M. Hudson, Division of Pathology, PHLS CAMR.

Table 2.3. Cosmid, plasmid and phage vectors.

Vector	Relevant characteristics	Source/Ref.
Cosmid pLAFR1	21.6 kb, tetracycline resistant	Friedman <i>et al.</i> (1982).
Cosmid p16	pLAFR1 clone of <i>B. pertussis</i> library	Brownlie <i>et al.</i> (1986).
Plasmid pIC20H	2.7 kb, ampicillin resistant	Marsh <i>et al.</i> (1984).
Plasmid pIC20H containing 0.58 kb insert	<i>B. pertussis</i> BscI 0.58 kb fragment from p16 cloned into pIC20H	This study.
Plasmid pKK1001B	pBR322 with a 8.8 kb <i>S. typhimurium</i> DNA insert	Dr K. Kutsukake ^a .
M13 mp18, mp19		Yanisch-Perron <i>et al.</i> (1985)

^a Hiroshima University, Japan

chemically defined Basal medium (Stainer and Scholte, 1971) containing 2-6-0-dimethyl- β -cyclodextrin (Imaizumi *et al.*, 1983), growth supplement and 1% (w/v) casamino acids (Section 2.2.). Filter-sterilised growth supplements and autoclaved casamino acids were added to autoclaved broth immediately prior to use. *B. pertussis* was grown for c. 48 h and the other *Bordetella* for c. 36 h until the cultures were turbid. All cultures were grown under X-mode conditions to produce virulent organisms. *B. pertussis* strain Taberman was also grown under C-mode conditions (Lacey, 1960) by replacing the NaCl in the media with MgSO₄ (Section 2.2).

Other species were grown at 37 °C on nutrient agar, or with agitation in liquid culture, either in Nutrient broth or L-broth (Section 2.2). *E. coli* TG1 was grown on minimal media agar (Section 2.2).

The antibiotics used are listed below.

Antibiotic	Working concentration	Stock solution
Ampicillin	50 µg/ml	25 mg/ml in distilled water.
Erythromycin	25 µg/ml	10 mg/ml in ethanol.
Tetracycline	10 µg/ml	5 mg/ml in 50% ethanol. Stored in the dark.
Cephalexin	40 µg/ml	Stored in fridge.

All antibiotics in solution were sterilised by filtration using a 0.22 micron (Millipore) disposable filter and stored in aliquots at -20 °C.

2.6. MICROBIOLOGICAL TECHNIQUES.

2.6.1. Determination of phase/mode of *Bordetella*.

The phase/mode of each strain grown for chromosomal DNA preparation was checked as follows:

2.6.1.a. Growth on selective agar.

Phase IV strains but not phase I strains of *B. pertussis* are able to grow on nutrient agar and BGA (Section 2.2.) containing erythromycin (2.5 µg/ml).

2.6.1.b. Haemolysin assay.

Strains were streaked to single colonies on BGA plates overlaid with BGA containing 20% blood (Section 2.2). After incubation for 5 days at 37°C, haemolytic activity was evident as a clear zone of haemolysis around the colonies.

2.6.1.c. Haemagglutination.

Defibrinated horse red blood cells (Tissue Culture Services Ltd. Buckingham) were washed three times in phosphate-buffered saline, PBS. Packed cells were diluted to 2% (v/v) in PBS and 100 µl volumes were mixed with 100µl of bacterial cells (grown in liquid culture) in the U-shaped wells of plastic microtitre trays (Nunc). These were covered and left overnight at room temperature. Haemagglutination was apparent when the red blood cells formed a "shield" of cells over the bottom of the wells, and lack of haemagglutination was shown by the formation of a characteristic pellet at the bottom of the well.

2.6.1.d. Motility.

Motility was tested by taking a loopful of *Bordetella* cells (grown overnight on BGA) and mixing the cells with 1ml of PBS to form a suspension,

followed by examination under phase contrast microscopy (Leitz Dialux microscope, 1,250 x magnification).

A summary of the phenotypic properties of the *Bordetella* strains used in this study is given in Table 2.4.

2.6.2. Transformation of *E.coli*.

Strains of *E.coli* were transformed and transfected (with plasmid and M13 DNA respectively) essentially as described by Cohen *et al.* (1972).

2.6.2.a. Preparation of competent cells.

E. coli JM83 was used for plasmid transformations and *E. coli* TG1 for phage M13 transfections (the F pilus of TG1 enabled binding of the M13 phage). TG1 was grown on minimal agar (Section 2.2) before growing overnight in liquid culture. A 0.5 ml sample of an overnight culture of the desired *E.coli* strain was used to inoculate 50 ml of prewarmed L-broth in a 250 ml conical flask. Cells were grown at 37 °C with vigorous aeration until the OD₄₅₀ of the culture reached 0.6 and the culture was then chilled on ice for 20 min. Cells were then harvested by centrifugation (3000 x g, 10 min, 4 °C) and the supernatant fluid discarded. The bacterial pellet was resuspended in 25 ml of cold 0.1 M MgCl₂ and immediately harvested as before. The cell pellet was then resuspended in 2.5 ml of cold 0.1 M CaCl₂ and the suspension was kept on ice for 2 h to make the cells competent.

2.6.2.b. Transformation/transfection.

Transformation/transfection was achieved by adding the transforming DNA (1 µg in a volume of 1-10 µl) to 100 µl of the competent cells and storing on ice for 30 min. The cells were then subjected to a heat shock by incubating them for 2 min at 42°C before returning them to the ice for 30 min. Transformed cells

Table 2.4. Phenotypic characteristics of the *Bordetella* strains used in this study.

Strain	Growth on Nutrient Agar	Growth on BG and erythromycin	Haemolysis	HA
<i>Bordetella pertussis</i> :				
Taberman	-	-	+	+
Taberman C-mode	-	-
Tohama	-	-	+	+
Wellcome 28,W28	-	-	+	+
Arg 40	-	-	+/-	+
Arg 41	-	-	+	+
L84 I	-	-	+	+
L84 IV	+	+	-	-
D30042 I	-	-	+	+
D30042 IV	+	+	+/-	+/-
134 I	-	-	+	+
134 IV	+	-	+/-	-
11615 IV	+	+	-	-
364 IV	+	+	-	-
M2 I	-	-	+/-	+
<i>Bordetella parapertussis</i> :				
5952	+	-	+	+/-
10520	+	-	+	+/-
<i>Bordetella bronchiseptica</i> :				
452	+	+	+/-	+/-
AS1 I	+	+	+	-
AS1 III	+	+	-	-
276 I	+	+	+/-	+
276 III	+	+	-	-
FW5 I	+	+	-	+
FW5 III	+	+	-	-
<i>Bordetella avium</i> :				
4148	+	...	-	...
4480	+	...	+	...

B. pertussis and *B. parapertussis* strains were found to be non-motile whereas all *B. bronchiseptica* strains were motile, *B. avium* strains were not tested for motility.

+ = positive for phenotype, +/- = weak for phenotype, - = negative for phenotype, ... = not tested for phenotype, HA= haemagglutination.

were diluted ten times in pre-warmed L-broth for expression of antibiotic resistances prior to plating on agar selective plates.

For M13 DNA transfection, the expression step was not necessary and cells were added directly to soft agar overlays after the heat shock. Prewarmed 25 ml plastic test tubes containing 3 ml molten H-top agar (Section 2.2), 80 μ l of X-Gal (20 mg/ml in dimethylformamide) and 40 μ l of IPTG (40 mg/ml in water) were held at 45 °C. Transfected TG1 cells and 0.2 ml of exponentially-growing *E. coli* TG1 cells were added to the tubes and gently mixed. This mixture was immediately poured as an overlay onto 2 x YT agar plates (Section 2.2). After the overlay had set the plates were inverted and incubated at 37 °C overnight.

2.7. DNA MANIPULATION TECHNIQUES.

Section 2.3. gives details of preparation of buffers and solutions mentioned but not described below.

2.7.1. Chromosomal DNA preparation (adapted from Marmur, 1961).

Bordetella strains were grown on BGA plates and a large inoculum (loopful of cells) was used to inoculate 200 ml of Basal medium (Section 2.2). The cultures were grown for 36-48 h, until turbid, and the cells harvested by centrifugation at 10,000 x g for 10 min at 4°C. The pellet was resuspended in saline EDTA, pH 8.0 (Section 2.3) (c. 10 ml per 2.5 g wet weight of cells). Lysozyme (Sigma) was added to give a final concentration of 1 mg/ml and the solution incubated with mixing at 37 °C for 30 min. The cells were lysed by the addition of TE buffer (Section 2.3) (4 ml to 20 ml suspension), proteinase K (Sigma, final conc 0.1 mg/ml) and SDS (final conc. 2 % w/v). The mixture was incubated at 37 °C for 1 h until lysis occurred, after which time the solution was de-proteinised by adding 0.33 volume of freshly-prepared sodium perchlorate (66.5% w/v in TE buffer) with gentle mixing. An equal volume of TE-saturated phenol/chloroform, 1:1 (Section 2.3), was then added and mixed until a stable

creamy emulsion was formed. The mixture was centrifuged (5,000 x g for 10 min), the clear upper layer removed for retention and extracted twice with chloroform:isoamyl alcohol (24:1 v/v). To precipitate the DNA from the solution, two volumes of ethanol (stored at -20 °C) was overlaid and the DNA gently spooled onto a glass rod, briefly air dried and then dissolved in 0.1 x TE buffer. To ensure the DNA was fully dissolved it was left overnight at 4 °C then stored at -20 °C. The DNA was treated with DNase-free RNase (BCL) final concentration 20 µg/ml before digestion with restriction enzymes and analysis by agarose gel electrophoresis.

2.7.2. Large scale cosmid/plasmid DNA isolation from *E. coli*.

This protocol was based on the method of Clewell and Helinski (1969). A single bacterial colony from an overnight plate was inoculated into 150 ml of L-broth (plus antibiotics where appropriate) and incubated at 37 °C for approximately 20 h with moderate shaking (250 rpm) in a conical flask. The cells were harvested by centrifugation (10,000 x g, 15 min, 4 °C) and resuspended in 2.6 ml 25% sucrose, 0.05 M Tris-HCl (pH 8.0). The cell suspension was transferred to a cold polypropylene centrifuge tube for the addition of 0.4 ml of a freshly prepared lysozyme solution (20 mg/ml in 0.25 M EDTA, pH 8.0) and swirled intermittently on ice for 5 min. Then, 2.6 ml of 0.25 M EDTA (pH 8.0) was added and swirled intermittently on ice for 5 min before the rapid addition of 4 ml of the lysing solution (1% (w/v) Brij 58, 0.4% (v/v) sodium deoxycholate in 0.01 M Tris, 0.001 M EDTA, pH 8.0). The solution was then drawn into and expelled three times from a 10 ml pipette to ensure even mixing and then left on ice for 20-30 min. The cell debris and the bulk of the chromosomal DNA was removed by centrifugation (10,000 x g, 45 min, 4 °C). The watery supernatant (cleared lysate) was carefully decanted into a fresh tube and the volume measured accurately with a 10 ml pipette. Covalently-closed circular plasmid DNA was separated from chromosomal DNA and nicked and linear plasmid

DNA by centrifugation in caesium chloride-ethidium bromide density gradients. Solid caesium chloride was added at a concentration of 1 g/ml of cleared lysate. After this, 0.2 ml ethidium bromide solution (10 mg/ml in distilled water) was added and the solution transferred to a Ti50 polyallomer ultracentrifuge tube (Du Pont/Sorvall "quick-seal"). Tubes were balanced to within 50 mg in pairs by the addition of caesium chloride solution (prepared by dissolving 1.0 g CsCl in 1 ml TES; Section 2.3). The upper reaches of the inside wall of each tube were dried and liquid paraffin was added to fill the tube. The tubes were then balanced to within 50 mg before sealing using the Du pont/Sorval tube crimping system. The balance of each pair of tubes was then confirmed and the tubes centrifuged (140,000 x g, 36-48 h, 20 °C). After centrifugation the bands of DNA in the tubes were visualised with a UV long wave transilluminator (Black-ray Ultraviolet Products Inc., Cambridge, U.K.). Initially, the tube was pierced at the top with a hypodermic needle. Subsequently, 23-gauge hypodermic needle was used to pierce the tube approximately 1cm below the lower (plasmid) band and the solution was allowed to drip out through the needle. The fraction containing the plasmid was collected and the ethidium bromide was removed by extracting three times with isoamyl alcohol (equilibrated with TE saturated with CsCl). The clear, lower phase containing the DNA solution was then transferred to prepared dialysis tubing (1/4", Gibco-BRL, Section 2.3.) and the CsCl was removed by dialysis against 1 litre volumes of TE buffer (Section 2.3.) (4 °C for 1 h, 3 h and overnight).

2.7.3. Phenol purification of plasmid DNA.

Deproteinization of DNA samples was achieved by extracting samples with an equal volume of phenol, pre-equilibrated with 100 x TE buffer (Section 2.3). The DNA/phenol solution was mixed for 1 min on an Eppendorf shaker, left to stand for 5 min and then shaken again for 1 min. The phases were then separated by centrifugation (13000 x g, 2 min) and the aqueous (top) phase

transferred to a fresh tube. Traces of phenol were removed by extraction 3 times with a double volume of water-saturated diethyl ether. Any residual ether was removed by incubation at 55 °C for 10 min. The purified DNA sample was then ethanol precipitated.

2.7.4. Ethanol precipitation of DNA.

For routine precipitation of DNA, 0.1 volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of chilled absolute ethanol were added to samples. Following incubation for at least 1 h at -20 °C or 15 min. at -70 °C, DNA was recovered by centrifugation (13000 x g, 4°C, 10 min). The supernate was carefully decanted and discarded. The DNA pellet was then washed in 70% (v/v) ethanol, centrifuged as above (2 min only), dried under vacuum and resuspended in an appropriate volume of TE buffer.

2.7.5. DNA concentration and purity.

The DNA concentration and the level of protein contamination was determined by diluting the DNA in TE buffer (Section 2.3.) and measuring the optical density at 260 nm and 280 nm in a quartz cuvette in a spectrophotometer (Pye Unicam, PU, 8600, UV/Vis). An optical density of 1.0 at 260 nm is equivalent to a DNA concentration of 50 µg/ml, for double stranded DNA, 40 µg/ml for single stranded DNA and RNA, and approximately 20 µg/ml for single stranded oligonucleotides (Sambrook *et al.*, 1989). Protein or phenol contamination was considered negligible if the ratio OD_{260}/OD_{280} was 1.7 or greater.

Alternatively the concentration of a DNA sample was estimated visually on a U.V. transilluminator after separating samples by electrophoresis on agarose gels alongside samples of lambda of known concentration for direct comparison of band intensities after staining with ethidium bromide and photographing.

2.7.6. Digestion of DNA with restriction endonucleases.

All restriction endonucleases were purchased from either Boehringer Mannheim (BCL), Gibco-BRL, New England Biolabs or Northumbria Biologicals Ltd. (NBL) .

Restriction enzymes were used according to the manufacturer's instructions except that an excess of enzyme (2-10 x) was used routinely with an incubation of 3 h. For chromosomal digests, an excess of enzyme (15 x) was used with an extended digestion time of 5 h to ensure that there were no partial digestion products. If digested DNA was to be used for ligation, endonuclease activity was destroyed by heat inactivation at 65 °C for 10 min.

Restriction enzymes used in this study are detailed in Table 2.5. *ClaI* and *BscI* are isoschizomers which cleave the same restriction site. Double digestions with *StuI* and *BscI* used the *BscI* buffer as this was more compatible for both enzymes than the *ClaI* buffer. DNA obtained from *dam*⁺ *E.coli* strains was partially resistant to cleavage by *ClaI*. Further methylase sensitive enzymes used in this study are detailed in Table 2.6.

2.7.7. Size markers for DNA electrophoresis.

To prepare lambda *HindIII* markers, 20 µg of lambda DNA was digested with *HindIII* at 37 °C for 3 h and either used directly or stored at -20 °C. Before use, the sample was heated at 65 °C for 5 min and rapidly cooled to separate annealed *cos* sites. Routinely 1.0 µg of *HindIII*-digested lambda DNA was mixed with 1/4 volume of agarose gel-loading dye and loaded on a electrophoresis gel. This quantity of lambda DNA allowed visual identification of all bands.

The lambda *HindIII* fragment sizes (kb) are 23.1, 9.4, 6.5, 4.4, 2.3, 2.0, 0.56, 0.12.

The 1 kilobase (kb) DNA ladder was suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb. The kb ladder was

Table 2.5. Restriction enzymes.

ENZYME	CLEAVAGE SITE
	5' ----->3'
<i>Bam</i> HI	G' GATCC
<i>Ban</i> III	AT' CGAT
^a <i>Bbv</i> I	GCAGC(N) ₈ '
<i>Bgl</i> II	A' GATCT
<i>Bsc</i> I	AT' CGAT
<i>Cla</i> I	AT' CGAT
<i>Eco</i> RI	G' AATTC
<i>Eco</i> RV	GAT' ATC
<i>Hind</i> III	A' AGCTT
<i>Kpn</i> I	GGTAC' C
<i>Mbo</i> I	' GATC
<i>Nru</i> I	TCG' CGA
<i>Pst</i> I	CTGCA' G
<i>Sac</i> I	GAGCT' C
<i>Sal</i> I	G' TCGAC
<i>Sau</i> 3A	' GATC
<i>Sma</i> I	CCC' GGG
<i>Sph</i> I	GCATG' C
<i>Stu</i> I	AGG' CCT
<i>Xba</i> I	T' CTAGA
<i>Xho</i> I	C' TCGAG

^a*Bbv*I: cleavage occurs 3' of the last nucleotide (N) in the recognition sequence above.

': signifies a recognition cut site.

Table 2.6. Methylase sensitive enzymes.

ENZYME	CLEAVAGE SITE	METHYLATION SENSITIVITY
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Dam methylation sensitivity:

<i>DpnI</i>	GA ^{m6'} TC	Active only if Dam methylation present at cleavage site.
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<i>MboI</i>	' GATC	Blocked by Dam methylation.
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<i>Sau3A</i>	' GATC	Not blocked by Dam methylation.
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<i>BscI</i>	AT'CGAT	Sensitive to Dam methylation when recognition sequence preceded by a 5' guanine or followed by a 3' cytosine.
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<i>ClaI</i>	AT'CGAT	Does not cleave DNA when either the A or C residue is N ⁶ -methyladenine or N ⁵ -methylcytosine respectively.
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CCGG Methylation sensitivity.

<i>MSpI</i>	C' CGG	Not blocked by CCGG methylation at N ⁴ or N ⁵ position of internal cytosine (but is by N ⁵ methyl cytosine at the 5' C residue).
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<i>HpaII</i>	C' CGG	Blocked by CCGG methylation at N ⁴ or N ⁵ position of internal cytosine.
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Dcm methylation sensitivity:

<i>BstNI</i>	CC' (A/T)GG	Not blocked by Dcm methylation.
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<i>EcoRII</i>	'CC(A/T)GG	Blocked by Dcm methylation.
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': signifies recognition sequence cleavage site.

Dam methylation = methyl group transferred from S-adenosyl-methionine to N⁶ position of the adenine residues in sequence GATC. Dcm methylation = methylation of internal cytosine residues in sequence CCAGG.

diluted to use, kb ladder: dye : sterile distilled water 100:100:300 μ l; 1.0 μ g per track produced distinct bands.

The 1 kilobase DNA ladder fragment sizes (kb) are 12.0, 11.0, 10.0, 9.0, 8.0, 7.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.6, 1.0, 0.5.

2.7.8. Agarose gel electrophoresis.

Electrophoresis through agarose was used to separate, identify and purify DNA fragments. The horizontal gel electrophoresis systems employed were obtained from Gibco-BRL (H4 and Horizon 58 systems). Routine analytical gels were made in 1 x TBE buffer (Section 2.3.) using agarose (type 1, low EEO, Sigma) at a concentration of 1% w/v (in 1% agarose there is a linear relationship between \log_{10} size and mobility for DNA fragments between 7 kb and 0.5 kb). To separate smaller fragments (0.1-0.5 kb) the agarose concentration was increased to 2% w/v. Low gelling temperature agarose (Type XI, Sigma) was also used in preparative gels for cloning and probe preparation. Agarose was dissolved in the buffer by heating in a microwave oven, then cooled to 50 °C before pouring into plastic gel casting trays. Usually, 100 ml and 300 ml of molten gel was used for the model H5 midi and H4 systems respectively. After the gel had set (c. 30 min, at 4 °C), the comb was removed and the gel submerged in 1 x TBE gel running buffer. Samples were mixed with a 0.1 volume of agarose Gel Loading dye (Section 2.2) prior to loading into the wells. Gels were subjected to electrophoresis (15 V/cm for 5 h or 1-5 V/cm overnight) and stained by adding ethidium bromide (0.2 μ g/ml) to the electrophoresis buffer for overnight electrophoresis or otherwise placed in a solution of ethidium bromide (0.5 μ g/ml in water) for 30 min and then rinsed in distilled water. DNA was visualised by UV illumination on a transilluminator (Black-ray Ultraviolet Products, Cambridge, UK) and photographed with a Polaroid camera (MP4 land camera system) through a red filter using Polaroid 667 black and white film (ASA 3000). Normally, an aperture setting of F8 and exposure times of 1-4

seconds were sufficient. The size of the fragments were calculated by reference to the DNA markers. If precise measurements were needed the distance travelled by the marker fragments from the origin was plotted against log size (in kb) on semi-log paper to produce a calibration curve for that gel. The size of the test DNA was determined from its mobility by comparison with the calibration curve.

2.7.9. Purification of DNA from agarose.

DNA was purified from agarose using a Geneclean II kit according to the manufacturers specifications (obtained from Stratech Scientific Ltd, Luton, UK). The band containing the DNA to be purified was excised from the agarose gel with a scalpel, weighed, and placed in a microfuge tube. NaI stock solution (6M) three times the weight of the gel slice was then added. The tube was incubated at 50°C for 5 min with intermittent mixing to dissolve all the agarose. For agarose gels made with TBE buffer, 0.5 volume of TBE Modifier and 4.5 volumes of NaI were then added. For DNA content < 5 µg, 5µl of vortexed glassmilk suspension (silica matrix in water) was added. The solution was incubated on ice for 5 min and mixed every 1-2 min to allow binding of the DNA to the silica matrix. The microfuge tube was then centrifuged for approximately 5 s and the supernate discarded. The pellet was washed 3 times with 450 µl ice cold "New Wash" (NaCl/ethanol/water). The DNA was eluted from the glassmilk by resuspending it in TE buffer and incubating at 50 °C for 3 min. The tube was centrifuged for 30 s and the supernatant containing the DNA removed. A second elution was performed if a high recovery was required. The eluted DNA was used immediately or stored at -20°C.

2.7.10. Subcloning.

The vector plasmid pIC20H DNA was digested overnight with *Cla*I at 37°C in a total volume of 20 µl, and 1 µl was tested on an electrophoretic gel to

ensure it was fully digested. The digested DNA was then heated at 70°C for 15 min to inactivate the restriction enzyme. If the vector was to be dephosphorylated to prevent self ligation calf intestinal phosphatase (Gibco-BRL: at 5 units per μg of DNA) was added and the tube incubated at 37°C for 1 h. Afterwards EDTA was added to a final concentration of 5 mM and the mixture then heated at 75°C for 10 min to inactivate the enzyme. The vector and insert DNA were then subjected to electrophoresis on a LMP agarose gel and the fragments purified with Geneclean prior to ligation.

2.7.11. Ligation.

Ligations were performed in a 0.5 ml microfuge tube using 100 ng vector and an equimolar amount of insert DNA in 1 x ligase buffer (5 x ligase buffer supplied with the T4 DNA ligase by the Gibco-BRL manufacturers). If less insert DNA was available, 50 ng of vector was used and a maximum volume of insert. T4 DNA ligase was added at a concentration of approximately 0.1-1.0 unit per μg of vector DNA and the mixture incubated overnight at 15 °C.

2.7.12. Southern blotting.

The transfer of electrophoretically-separated DNA fragments from a gel to a solid support was first reported by Southern (1975). The technique involves blotting the DNA from an agarose gel to a nitrocellulose or nylon membrane, followed by the detection of specific immobilised sequences by hybridisation.

2.7.12.a. Alkaline Southern blotting with Zeta-Probe nylon membrane.

"Zeta-Probe" membranes (Bio-Rad) bind DNA irreversibly and quantitatively in alkaline solution eliminating the need for post-transfer fixation and resulting in an increase in sensitivity. Transfer of DNA from agarose gels to the "Zeta-Probe" nylon membrane was achieved using alkaline solutions and capillary transfer (Reed and Mann, 1985). Before transfer, ethidium bromide-

stained gels were visualised under UV illumination and photographed. In order to facilitate the transfer of large DNA fragments, the DNA was nicked by partial depurination by soaking the gel in c 500 ml 0.25 M HCl for 15 min. Gels were subsequently rinsed thoroughly with water. Four sheets of Whatman 3MM filter paper were cut and placed on a glass plate so that they overhung the bottom of the gel tray by 5 cm on each end (approximately 20 x 40 cm for a gel size of 20 x 20 cm). They were placed on an inverted gel casting tray in the bottom of a deep dish and the filter paper was then saturated with 0.4 M NaOH, as the neutralising/denaturing agent. Bubbles were removed by repeatedly rolling a glass pipette over the saturated filter paper, pouring enough NaOH into the deep dish so that the filter paper wick ends were immersed in NaOH. More NaOH was poured onto the filter paper wick to saturate it, then the gel carefully placed on the 3MM paper, again making sure that no bubbles were trapped beneath the gel. The gel was then covered with a small amount of NaOH. Saran Wrap plastic film was placed over the entire gel/filter paper stack and a window cut out allowing the gel to emerge. The Zeta-Probe membrane was moistened in distilled water and then lowered onto the gel surface making first contact in the centre, and then out to the edges. The filter surface was carefully flooded with NaOH, making sure that no bubbles were present between the gel and the membrane. Two pieces of filter paper were cut exactly to the size of the gel. A sheet of pre-cut filter paper was moistened with water and placed on the membrane/gel stack, then this was repeated with a second sheet. Any bubbles were removed from beneath each layer of filter paper. A stack of pre-cut blotting paper (QuickDraw, Sigma) was placed on the filter paper/Zeta-Probe membrane/gel stack and covered with a glass plate and a weight of c. 250 g to keep the blotting paper in intimate contact with the gel and with one another. The transfer was allowed to proceed overnight; after transfer the stack of blotting paper was removed and the orientation of the gel marked on the membrane. The Zeta-Probe membrane was gently peeled from the surface of

the gel and rinsed in 2 x SSC (Section 2.3) and air dried. The dried membranes were stored at room temperature. Membranes could be kept indefinitely in this condition. To confirm satisfactory transfer of the DNA, the gel was restained with ethidium bromide and visualised under UV light to ensure no DNA remains in the gel.

2.7.12.b. High salt Southern blotting with Hybond-N nylon membrane.

Another transfer method used high salt concentration Southern blotting with the same capillary transfer as in 2.8.12.a. The gel was treated with 0.25 M HCl and briefly rinsed in distilled water. However, before placing the gel in the blot it was soaked for 30 min in 250 ml denaturing solution (0.5 M NaOH, 1.5 M NaCl) and rinsed again in distilled water. The gel was then washed in neutralising solution (3 M NaCl, 0.5 M Tris-HCl, pH 7.4) for 30 min. The DNA was transferred to Hybond-N membrane (Amersham) with 20 x SSC. The nylon blotting membrane and 3MM filter paper cut to the size of the gel were soaked in 3 x SSC. After transfer, the membrane was rinsed briefly in 3 x SSC, blotted dry with filter paper and allowed to dry at room temperature. The membrane was then wrapped in Saran Wrap plastic film so that only a single layer covered the side of the membrane that was in contact with the gel, the "DNA side". The wrapped membrane was placed on the U.V. transilluminator, for c. 2 min DNA side down, to allow the U.V. light to covalently crosslink the DNA to the membrane. The membranes were stored at 4°C.

2.7.13. Radiolabelling of size markers for Southern blots.

Lambda *Hind*III fragments were labelled at their 3' end and 1.0 kilobase ladder DNA marker (1kb) was labelled by filling in the 3' recessed ends using DNA polymerase. To 10 µl (about 10 µg) of lambda *Hind*III digest (or 1 kb ladder) was added 78 µl SDW, 10 µl of sequence buffer (Sequencing kit USB 70700, Section 2.4.20.c.), 1 µl (10 µCi) [α -³²P]dATP (400 Ci/mmol, Amersham

code PB 10164) and 1 μ l of the Klenow fragment of *E. coli* DNA polymerase (BRL). The reaction was left at room temperature for 15 min, loading buffer was added and the whole mixture divided between two agarose gel slots flanking the other DNA samples on both sides. Depending on the age of the ^{32}P -label, unlabelled DNA could be added to labelled material before loading to reduce the intensity of ^{32}P radiation.

2.7.14. Amplification of gene probes by the polymerase chain reaction (PCR).

PCR is a powerful technique based on primer-directed enzymic amplification of target DNA (Mullis and Faloona, 1987). Two oligonucleotide primers are designed from opposite strands of the DNA template so as to flank the target sequence. The 3' ends are orientated such that they point towards each other. Repeated cycles of template denaturation, primer annealing and polymerase-directed extension of the annealed primers, results in the exponential accumulation of the target sequence as defined by the 5' ends of both primers.

The GeneAmp PCR reagent kit (Perkin Elmer Cetus) was used to produce the PCR product. Both primers were separately diluted in TE buffer to a concentration of 600 pM. The reaction mixture in a 500 μ l microfuge tube contained: 1 x reaction buffer [10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.001% (w/v) gelatin]; 200 μ M each dNTP; 5 μ l of each diluted primer; and x μ l of SDW to result in a total volume of (90 μ l). Template DNA, 10 μ l (10 ng/ μ l) of plasmid DNA or 10 μ l (80 ng/ μ l) chromosomal DNA, and 0.5 μ l of recombinant *Taq* DNA polymerase ("AmpliTaq") were then added. The reaction mixture was overlaid with light mineral oil (Sigma) and subjected to automatic thermal cycling in a programmable thermal cycler (MJ Research Inc.). A thermal programme which worked well was:

1.	1.5 min	94 °C	Denaturation
2.	1.0 min	42 °C	Annealing

3.	1.5 min	72 °C	Extension
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The cycle stages 1-3 were repeated 25 times.

4.	4 min	72 °C	Final Extension
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5.	24h	4 °C	Cooling and Storage
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Controls where no template DNA was added were included in order to ensure that none of the reagents were contaminated by previous PCR reactions.

The PCR product was subjected to electrophoresis on a 1% low melting point (LMP) agarose gel (Sigma). 10 µl of each reaction mix was usually sufficient to visualise the PCR product. The relevant purified PCR DNA fragment was then excised from the gel and stored as in 2.4.15.a.

2.7.15. Synthesis of oligonucleotides.

Oligonucleotides were synthesised on an Applied Biosystems Model 380A DNA synthesiser by Mr K. Fantom and Mr R. Hartwell (Division of Biotechnology, PHLS CAMR). The samples were desalted by passage through a 10ml prepacked Sephadex G-25 (Pharmacia, PD-10) column.

The concentration of oligonucleotides was determined spectrophotometrically. The OD₂₆₀ was measured using a quartz cuvette in a spectrophotometer (Pye Unicam, PU 8600). A reading of 1.0 was taken as representing a concentration of 20 µg/ml for oligonucleotides (Sambrook *et al.*, 1989). Where necessary, oligonucleotides were diluted with TE and stored at -20°C.

2.7.16. Preparation of radioactively-labelled probes.

2.7.16.a. Random hexanucleotide labelling of gene probes.

This protocol was based on the method of Feinberg and Vogelstein (1984). In this procedure, denatured DNA is hybridised at a large number of

sites along the DNA with hexamer oligonucleotides that have random mixed bases at each position. These oligonucleotides serve as primers for DNA synthesis by Klenow polymerase in the presence of radionucleotides.

The Multiprime labelling system (Amersham) was used to label the DNA according to the manufacturers instructions. Restriction endonuclease-digested DNA was fractionated in a LMP agarose gel. The concentration of the DNA was estimated to ensure at least 250 ng of DNA was contained in the agarose band so that 25 ng could be extracted and used in the labelling protocol without concentrating the DNA. The band was excised cleanly with a scalpel blade, with the minimum amount of excess agarose, and transferred to a preweighed 1.5 ml polypropylene microfuge tube. SDW was added at a ratio of 3 ml per gram of gel, and placed in a boiling water bath for 7 min. to melt the gel and denature the DNA. If the DNA was not used immediately, the boiled samples were divided into aliquots and stored at -20 °C. On removal from -20°C, the DNA (25-100 ng, 1-10 µl) sample was then denatured by heating to 95-100 °C for 3 min in a boiling water bath (reboiling more than 3 times was avoided), and incubated at 37 °C for 10 mins. The following reagents from the Amersham kit were then added: 4 µl each of CTP,GTP,TTP, 5 µl buffer (reaction buffer containing: Tris-HCl (pH7.8), MgCl₂ and 2-mercaptoethanol), 5 µl primer (random hexanucleotides in an aqueous solution), SDW (the volume as appropriate for a final reaction volume of 50 µl), 5 µl [α -³²P]dATP (400 Ci/mmol, Amersham) and 2 µl Klenow fragment polymerase (1 unit per µl, Amersham). The reaction mixture was incubated overnight at room temperature overnight to ensure labelling to high specific activity. For use in a hybridisation reaction, the labelled DNA was denatured by heating to 95-100 °C for 5 min. The volume was made up to 200 µl with SDW and the probe used immediately by adding to the pre-hybridisation solution.

2.7.16.b. Radiolabelling of oligonucleotide probes.

Oligonucleotides that were to be used as probes were initially diluted to 100 ng/ μ l. They were then labelled at their 5' hydroxy termini by the action of T4 polynucleotide kinase. 2 μ l of the diluted oligonucleotide was added to a mixture containing 5 μ l aqueous [γ - 32 P]-dATP (3000 Ci/mmol), (10 Ci/ μ l), (Amersham), 2 μ l 10x kinase buffer (Section 2.3), 9 μ l distilled water and 20 units of T4 polynucleotide kinase (BCL, 10 units/ μ l). The reaction was allowed to proceed for 60 min at 37°C and then the probe was added directly to the pre-hybridisation mixture.

2.7.17. Hybridisation conditions.

Pre-hybridisation, hybridisations and washes were carried out in a rotor oven (Bachofen Ltd).

2.7.17.a. Low stringency hybridisation with Zeta-Probe membrane.

S. typhimurium DNA probes, multiprime-radiolabeled in agarose (Section 2.8.15.a), were hybridised to *Bordetella* DNA bound to Zeta-Probe. As different species were involved, low stringency hybridisation was used. Prehybridisation was initially at 42°C with agitation overnight in 20 ml of a solution containing 6.6 x SSC, 1% SDS, 5x Denhardt's solution (Section 2.3), 33% formamide and 10 μ g/ml denatured salmon sperm DNA (Sigma) (Sambrook *et al.*, 1989). The salmon sperm DNA and Denhardt's reagent acted as blocking agents to prevent non-specific attachment of the DNA probe to the membrane. The probe was placed in a boiling water bath for 5 min, and added to the pre-hybridisation mix as described above and the membrane again agitated at 42°C overnight. The membrane was washed twice in 5x SSC, 0.1% w/v SDS, for 30 min at 50°C and twice in 1 x SSC, 0.1% w/v SDS for 1 h at 50°C. The membrane was air dried and wrapped in Saran wrap before autoradiography (Kodak X-Omat AR, XAR-5 film). Sensitivity was increased by sandwiching the film and membrane

between two intensifying screens inside the light-proof cassette.

2.7.17.b. Hybond-N membrane.

Hybond-N membrane was used in initial work and, as both the DNA bound to the membrane and that used as a probe were from *B. pertussis*, higher stringency conditions were used. Prehybridisation solution consisted of 4 x SSC, 50% formamide, 4 x Denhardt's, 0.4% SDS, and 2 mg/ml salmon sperm DNA. After prehybridisation overnight at 42°C the labelled probe was added and hybridisation continued overnight at 42°C. Washes consisted of two at room temperature for 30 min with 2 x SSC, 0.1% SDS and two at 68°C for 1 h with 1 x SSC, 0.1% SDS.

For a lower stringency hybridisation, the formamide was reduced to 20 % and the final washes carried out at 50°C for 60 min with 2 x SSC and 0.1% SDS.

2.7.17.c. Oligonucleotide hybridisation.

Hybridisation temperatures varied between oligonucleotides, the temperature to be used was calculated as 5°C below the dissociation temperature as described by Suggs *et al.* (1981) using the formula: $T_d(^{\circ}\text{C}) = 2(A+T) + 4(G+C)$. This formula gives the temperature, in degrees centigrade, at which one half of oligonucleotide-genomic DNA duplexes are dissociated. In general, this formula is applicable for oligonucleotides of 10 to 21 nucleotides in length. The pre-hybridisation solution consisted of 20 ml of 6 x SSC, 5x Denhardt's solution, 0.5% SDS, (0.05% sodium pyrophosphate was included in some solutions to reduce background binding) and 10 µg/ml salmon sperm DNA and incubation was overnight at the same temperature calculated for the hybridisation. The labelled oligonucleotide was added to the prehybridisation solution and the hybridisation continued overnight at the same temperature. The membranes were washed three times in 6 x SSC, 0.1% SDS, at room temperature, and then finally for 30 min at the temperature used for the

hybridisation.

2.7.18. Autoradiography.

This was used for visualisation of radiolabelled fragments following both Southern hybridisation and DNA sequencing. After Southern hybridisation, the membrane was sealed in a plastic bag and placed in a X-ray cassette (Kodak X-Omatic) fitted with intensifying screens (regular). In the dark-room, a sheet of X-ray film, Kodak X-AR5 (high sensitivity), was placed next to the membrane and the cassette placed at -70 °C overnight or for as long as necessary up to two weeks. For sequencing gels, Kodak X-Omat S was placed next to the dried immobilised gel and the cassette placed at room temperature.

Film was processed manually or machine processed. Manual developing of the film involved submerging in developer (LX24 Kodak) for 5 min, draining, rinsing in stop solution (3% acetic acid) and submerging in fixer (Kodak Unifix) for 5 min, cleared by rinsing extensively in water and air drying. Machine processing used the Kodak X-Omat automatic film developer, Model M7B RP X-Omat.

2.7.19. Stripping probe from membrane for membrane reuse.

The membrane blot was incubated at 45 °C for 30 min in 0.4 M NaOH and then transferred to a solution of 0.1 x SSC, 0.1% (w/v) SDS, 0.2 M Tris-HCl (pH 7.5) and incubated for a further 15 min. After checking by autoradiography that the probe had been removed, the membrane was rehybridised with another probe.

2.7.20. DNA sequencing.

DNA sequencing was based on the "chain-termination" procedure first described by Sanger *et al.* (1980) and subsequently modified by Tabor and Richardson (1987). The DNA fragment to be sequenced was initially cloned into

the plasmid pIC20H, but, due to problems of compression of bands on the sequencing gel, the fragment was recloned into the replicative forms (RF) of the phage M13 cloning vectors mp18 and mp19 and fully sequenced. All these vectors contain multiple cloning sites in the alpha-peptide coding region of the beta-galactosidase gene. Insertion of foreign DNA into this polylinker sequence destroys active alpha-peptide synthesis, enabling the detection of recombinants in a suitable *E. coli* host on media containing the chromogenic substrate, X-gal (BCIG). Single-stranded template DNA was prepared from recombinant plasmids and phage particles and was used directly in the sequencing reaction. DNA sequencing involves the synthesis of a complementary DNA strand by DNA polymerase *in vitro*. Synthesis is initiated, in the presence of four dNTPs, from a short (15-30 nucleotides) oligonucleotide primer complementary in sequence to the plasmid or viral template DNA enabling annealing adjacent to the insert DNA sequence. Sequential extension of DNA using DNA polymerase forms a sequence complementary to the insert DNA. The addition of a radiolabelled dNTP enables visualisation of the newly synthesised DNA by autoradiography. The growing chain is terminated randomly by the incorporation of a 2', 3'-dideoxynucleotide triphosphate (ddNTP) in each of four reaction mixtures that will not support continued DNA synthesis. Since the sequence of each chain is determined by the template sequence, size fractionation of the reaction products by denaturing polyacrylamide gel electrophoresis enables the DNA sequence to be determined.

2.7.20.a. Subcloning of DNA into M13 mp18 and mp19.

Routinely, 50-100 ng of digested, dephosphorylated M13 vector (RF) was ligated (Section 2.8.11) with an *c.* equal molar quantity of compatible insert/target DNA. The subsequent ligation products were used to transfect competent *E.coli* TG1 (Section 2.6.2.b. Transfection).

2.7.20.b. Preparation of template DNA.

An overnight culture of *E.coli* TG1 in 2 x YT broth was diluted 1:100 with more pre-warmed broth. A 2 ml aliquot was infected with a single M13 plaque and incubated with vigorous aeration at 37 °C for 6 h. The culture was then transferred to a microfuge tube and centrifuged (13000 x g, 10 min). The supernatant liquid (c. 1.25 ml) was decanted into a fresh microfuge tube containing 250 µl 20% (w/v) PEG 6000, 2.5 M NaCl. The tube contents were thoroughly mixed on an Eppendorf shaker for at least 15 min before being centrifuged again (13000 x g, 10 min). The supernatant liquid was discarded by aspiration leaving the phage pellet in the tube. Care was taken to remove all traces of supernatant. The phage pellet was resuspended in 100 µl TE buffer and extracted with 50 µl TE-saturated phenol (Section 2.3). Following brief centrifugation (13000 x g, 2 min), the aqueous phase was transferred to a fresh microfuge tube and extracted twice with 0.5 ml aliquots of water-saturated ether (sterile distilled water: ether; 1:1). Finally, the sample was ethanol-precipitated (Section 2.8.4), washed with 70% ethanol, dried under vacuum and resuspended in 30 µl TE buffer and stored at -20°C.

2.7.20.c. Annealing of template with primer and sequencing reactions.

The Sequenase Version 2 DNA Sequencing Kit was used (70700, U.S. Biochemical, Corporation, Cambridge). Sequencing reactions were performed using "Sequenase" (a modified bacteriophage T7 DNA polymerase, USB). Templates were annealed with the appropriate oligonucleotide primer. Microfuge tubes containing 7 µl template DNA (approx. 1-2 µg), 1 µl oligonucleotide primer (2 ng/µl in TE) and 2 µl 5x Sequenase buffer (Sequenase kit: 200 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, 250 mM NaCl) were heated to 65 °C for 2 min and cooled slowly to room temperature to allow the primer to anneal to the template. Meanwhile, 2.5 µl aliquots of T,C,G and A termination

mixtures (80 μ M each dNTP, 50 mM NaCl, 8 μ M ddTTP/CTP/GTP or ATP) were dispensed into autoclaved capless 1.5 ml microfuge tubes positioned in centrifuge tube racks (Centra). When the annealings had cooled sufficiently, 5.5 μ l of a labelling master mix (1/5x TE buffer, 10 mM DTT, 1.5 μ M dTTP, 1.5 μ M dCTP, 1.5 μ M dGTP, 1.5 μ M [α -³⁵S]-thio-dATP (1000 μ Ci/mMol), 4 units Sequenase) was added to each annealing mixture and the tubes incubated at room temperature for 5 min (labelling step). Aliquots of 3.5 μ l of this reaction mix were then added to each termination mix prepared earlier and the tubes incubated at 37 °C for 5 min (termination step). Reactions were stopped by the addition of 4 μ l of formamide loading buffer (USB, Formamide 95%, xylene cyanol FF 0.05%, bromophenol blue 0.05% and EDTA 20 mM). Samples were denatured by heating (75 °C water bath, 2 min) prior to loading on denaturing polyacrylamide gels (Section 2.8.20.e).

2.7.20.d. Plasmid sequencing.

CsCl-purified plasmid DNA, 4 μ g, was denatured in 0.2 M NaOH (final concentration) and incubated at 37 °C for 30 min. The mixture was neutralised by adding 0.1 volumes of 2 M ammonium acetate (pH 4.5) and precipitated with two volumes chilled absolute ethanol. The ethanol was run down the side of the tube and mixture kept at -70 °C for 20 min. The DNA was pelleted (13000 x g, 4°C, 20 min), washed with 70% (v/v) ethanol and vacuum dried before being redissolved in 7 μ l of SDW, 2 μ l 1x Sequenase buffer and 1 μ l oligonucleotide primer at 0.2 ng/ μ l. The annealing step and sequencing reactions were done as described above for single-stranded DNA. To reduce the formation of secondary structures and compressions during plasmid sequencing with recombinant *Bordetella* GC-rich DNA, dGTP was replaced with dITP, although dGTP reactions were also done in parallel with dITP reactions.

2.7.20.e. Denaturing polyacrylamide gel electrophoresis.

The thin-gel system employed for the resolution of DNA sequencing reaction products was based on that described by Sanger *et al.* (1980). Two glass plates (20 x 50 cm), one of which had notches, were cleaned thoroughly with Pyroneg detergent and then rinsed with absolute ethanol. The notched (front) plate was then treated on one side with "repelcote" (2% dimethyl-dichlorosilane in 1,1,1-trichloroethane, BDH). Simultaneously, the back plate was treated with a mixture of 750 μ l 10% glacial acetic acid and 75 μ l silane (γ -(methyl-acryloxy)-propyltrimethoxysilane, Sigma) in 25 ml absolute ethanol. The plates were allowed to dry in a fume cupboard and polished before being sandwiched together (coated sides facing) with 0.35 mm plastic spacers along the 50 cm edges. The plates were secured with bulldog clips and the edges sealed using 2" packaging tape. Gradient gel mixes were made from 40 ml 0.5x TBE gel mix (A) and 7 ml 5x TBE gel mix (B) (Section 2.3). Polymerisation was initiated by adding 80 μ l of 25% (w/v) ammonium persulphate and 80 μ l TEMED to gel mix (A) and 14 μ l of each to gel mix (B). To pour gradients, 5 ml of A followed by 7 ml of B was taken up in a 10 ml wide-bore pipette together with two air bubbles to allow slight mixing. The contents were then gently expelled between the two glass plates, followed by the remaining A mix using a syringe. Care was taken to avoid any air bubbles. Finally, a plastic gel comb was placed at the top of the gel and the mixes allowed to polymerise. Routinely, 40 samples (10 x 4) were loaded on each gel. If the gel was not to be used the same day Saran Wrap was placed over the end of gel and comb to prevent it drying out.

2.7.20.f. Loading and running of sequencing gels.

Sequencing gels were prepared as described (section 2.8.20.e). Gel wells were carefully flushed out with 1x TBE running buffer immediately prior to loading approximately 2 to 3 μ l of sample per well. The order of loading was

T,C,G,A, for the reaction mixtures for each template sequenced. A constant current of 25 mA for c. 3.5 h was applied to the gels. After electrophoresis was complete, the gel sandwich was disassembled and the gel (still stuck to the back plate) was fixed by immersion in 10% glacial acetic acid, 10% methanol for 15 min. Gels were dried in an oven at 80 °C for at least 2 h before autoradiography with Kodak X-Omat S film.

2.7.20.g. Analysis and editing of sequence data.

Computer analyses of sequence data was performed with the aid of software supplied by DNASTAR Inc., Madison, Wisconsin, USA. Programmes included Mapseq, which displays the DNA sequence with enzyme restriction sites and translation products; Align, optimal alignment of two DNA sequences aligned by the method of Wilbur and Lipman (1983); Dotplot, two DNA sequences compared to produce a dot plot of similarities either graphically or as a listing; Nucscan, search of nucleotide sequence databanks: Genbank, Los Alamos, USA, and EMBL, Heidelberg, Germany, for DNA sequence homology; Proscan, search of protein database NBRF (National Biomedical Research Foundation) for amino acid sequence homology by the method of Lipman and Pearson (1985).

CHAPTER 3

CHARACTERISATION OF A *B. PERTUSSIS* DNA FRAGMENT PREVIOUSLY PROPOSED AS HOMOLOGOUS TO *SALMONELLA TYPHIMURIUM* HIN INVERTASE DNA.

3.1. Introduction.

S. typhimurium has a DNA invertase, Hin, which can cause DNA inversion that in turn controls flagellar antigen synthesis. Southern blots of *B. pertussis* DNA (strains 134 phase I, L84 phase I and phase IV) showed hybridisation to a *S. typhimurium* DNA probe containing the *hin* gene (Foxall *et al.*, 1990). This probe hybridised to a 15 kb *Eco*RI fragment in the *B. pertussis* chromosomal DNA. No variation was observed between different *B. pertussis* strains, modes or phases. The *S. typhimurium* DNA probe used in that work was a 8.8 kb *Eco*RI/*Bam*HI fragment of DNA from plasmid pKK1001B (Foxall *et al.*, 1990; Fig. 3.1). The plasmid consists of pBR322 vector containing an insert of 8.8 kb *S. typhimurium* DNA between the unique *Eco*RI-*Bam*HI sites. The *S. typhimurium* DNA contained the genes for the *hin* DNA invertase, inverted repeats, the promoter and the structural gene for the H2 flagellin. The nucleotide sequence of the *S. typhimurium* invertible region along with the flanking DNA sequence was published by Simon *et al.* (1980) and Silverman *et al.* (1981); the latter, longer sequence is shown in Fig. 3.2.

A more specific probe was produced by digesting pKK1001B with *B*scI and *Stu*I (MacGregor, 1990; Fig. 3.2.). Digestion of pKK1001B with *B*scI and *Stu*I produced four fragments: a doublet (~5.8 kb) and two smaller ones (~678 bp and ~620 bp). The 678 bp fragment consisted of 526 bp of the 573 bp of the *hin* gene (47 bp of the N terminus was absent in the fragment, see Fig. 3.2). It also included the distal domain of the enhancer for FIS (Factor for Inversion Stimulation, a sequence-specific DNA binding protein essential for inversion) and 152 bp of DNA not known to code for a protein (Szekely and Simon, 1983) at the 3' end (Fig. 3.2). A gene library of *B. pertussis* DNA (strain Taberman phase I, fimbrial serotype 0,3) in *Escherichia coli* had been previously constructed by Brownlie *et al.* (1986) using the broad host range cosmid vector pLAFRI (Friedman *et al.*, 1982). The cosmid library contained inserts of *B. pertussis* DNA with an average size of 24.9 kb. The Taberman DNA gene library

Fig. 3.1. Plasmid pKK1001B.

The plasmid, ~12.8 kb, consists of the plasmid pBR322 DNA (thick line) with a small *EcoRI-BamHI* fragment replaced by an 8.8 kb *S. typhimurium* LT2 chromosomal DNA fragment (thin line) in *H2* "on" orientation. The boxes with an internal cross represent the inverted repeats. Redrawn from Kutsukake (personal communication).

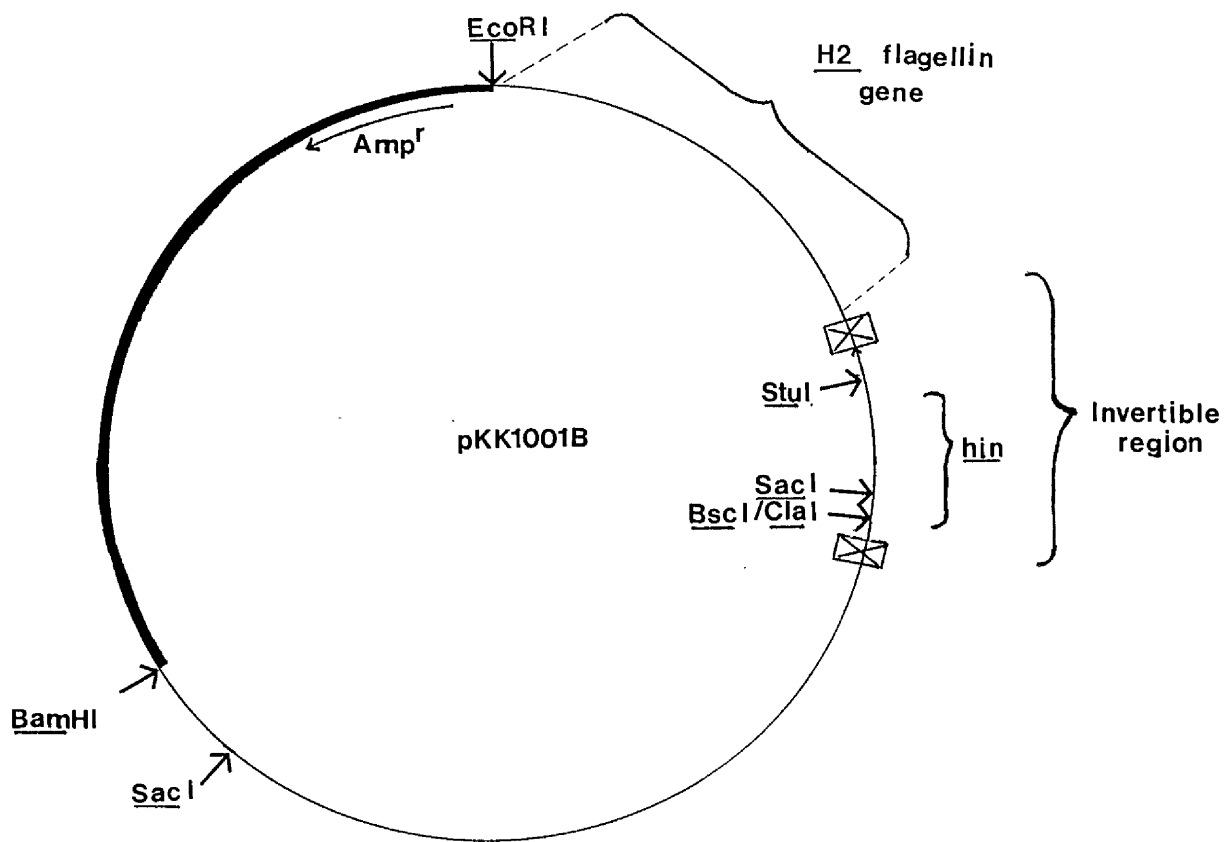


Fig. 3.2. Nucleotide sequence of the invertible region of *S. typhimurium* and adjacent DNA in *H2* "on" orientation (redrawn from Silverman *et al.*, 1981).

Predicted amino acid sequences are shown in all three reading frames for each DNA strand, (.) = stop codon. Areas of interest are marked, including the open reading frame (ORF) for the *hin* gene and *H2* flagellin gene and the restriction sites for *Cla*I (*Bsc*I), *Sac*I and *Stu*I. Inverted repeats left and right are marked IRL, IRR respectively. PCR oligo (1) and (2) identify sequences used to construct oligonucleotides to amplify *hin* gene from *S. typhimurium* DNA by PCR (Section 5.3).

The nucleotide sequences required for enhancer function are contained within a 60 bp segment of DNA which includes the areas marked (a) and (b) and the area between. Site (a) is the proximal domain and (b) the distal domain for FIS protein binding (Johnson and Simon, 1987).

ACGATTATGGTTCTTGAAAACCAAGGTTTTGATAAAGCAATCCTCCATGAGAAAAGCGACTAAAAATTCCTTATCTGATGTAAGGAGAAAATCA
TGCATAAATAACCAAGAACTTTTGGTTCCAAAAACTATTCGTTAGGAGGTACTCTTTTCGCTGATTTTAAGAAGGAATAGACTACATTTCTCTTTTAGT

100

t i r y w f l k t k v f d k a i l h e k s d . n s s l s d v k e k i
r f i g s . k p r f l i k q s s m r k a t k i l p y l m . r r k s
d l l v l e n q g f . . s n p p . e k r l k f f l i . c k g e n h
i . q n k f v l t k s l a i r w s f l s . f e e k d s t f s f i
r n i p e q f g l n k i f c d e m l f a v l i r g . r i y l l f d
s k n t r s f w p k q y l l g g h s f r s f n k r i q h l p s f .

begin
ORF
hin
invertase

-----a-----BscI/ClaI-----b-----
TGGCTACTATTGGGTATATTCGGGTGTCAACAATTGACCAAAATATCGATTACAGCGTAATGCGCTTACTAGTGCAAATTGTGACCGCATTTTGAAGA
ACCGATGATAACCCATATAAGCCACAGTTGTTAACTGGTTTTATAGCTAAATGTCGCATTACGCGAATGATCACGTTTAACTGGCGTAAAAACTTCT

200

hin ORF

→ a t i g y i r v s t i d q n i d l q r n a l t s a n c d r i f e d
w l l l g i f g c q q l t k i s i y s v m r l l v q i v t a f l k t
g y y w v y s g v n n . p k y r f t a . c a y . c k l . p h f . r
m a v i p y i r t d v i s w f i s k c r l a s v l a f q s r m k s s
h s s n p i n p h . c n v l i d i . l t i r k s t c i t v a n k f
p . . q t y e p t l l q g f y r n v a y h a . . h l n h g c k q l

CCGTATCAGTGGCAAGATTGCAAACGCCCGGCTGAAACGGGCGTTAAAGATGTAAATAAAGGCGATACTCTTGCTGCTGGAAATTAGACAGACTG
GGCATAGTCACCGTTCTAACGTTTGGCGGGCCGGACTTTGCCCGCAATTTACATACATTTATTTCCGCTATGAGAACAGCAGACCTTTAATCTGTCTGAC

300

→ r i s g k i a n r p g l k r a l k y v n k g d t l v v w k l d r l
v s v a r l q t a p a . n g r . s m . i k a i l l s s g n . t d w
p y q w q d c k p p r p e t g v k v c k . r r y s c r l e i r q t g
r i l p l i a f r g p r f r a n f y t f l p s v r t t q f n s l s
v t d t a l n c v a g a q f p r . l i y i f a i s k d d p f . v s q
g y . h c s q l g g r g s v p t l t h l y l r y e q r r s i l c v

SacI

GGCCGTAGCGTGAAAAATCTGGTGGCGTTAATATCAGAATTACATGAACGTGGAGCTCAGCTCCATTCTTTAACCGATAGTATTGATACAGTAGCGCGA
CCGGCATCGCACTTTTAGACCACCGCAATTATAGTCTTAATGTACTTGACCTCGAGTGAAGGTAAGAAATGGCTATCATAACTATGGTCATCGCGCT

400

→ g r s v k n l v a l i s e l h e r g a h f h s l t d s i d t s s a m
a v a . k i w w r . y q n y m n v e l t s i l . p i v l i p v a r
p . r e k s g g v n i r i t . t w s s l p f f n r . y . y q . r d
p r l t f f r t a n i d s n c s r p a . k w e k v s l i s v l l a
a t a h f i q h r . y . f . m f t s s v e m r . g i t n i g t a r
p g y r s f d p p t l i l i v h v h l e s g n k l r y y q y w y r s

TGGGGCGATTCTTTTTTCATGTAATGTGAGCACTGGCCGAGATGGAGCGAGAATTAATCGTCGAGCGAACCTTGCCGGACTGGCTGCCGCCAGAGCGCA
ACCCCGCTAAGAAAAAAGTACATTACAGTCGTGACCGGCTCTACCTCGCTCTTAATTAGCAGCTCGCTTGGGAACGGCCTGACCGACGGCGGTCTCGCGT

500

→ g r f f f h v m s a l a e m e r e l i v e r t l a g l a a a r a q
w g d s f f m . c q h w p r w s e n . s s s e p l p d w l p p e r k
g a i l f s c n v s t g r d g a r i n r r a n p c r t g c r q s a
i p r n k k . t i d a s a s i s r s n i t s r v r a p s a a a l a c
h p s e k k m y h . c q g l h l s f . d d l s g k g s q s g g s r
p a i r k e h l t l v p r s p a l i l r r a f g q r v p q r w l a

AGGACGACTGGGAGGGCGCCCTCGGGCGATCAACAAACATGAACAGGAACAGATTAGTCGGCTATTAGAGAAAGGCCATCCTCGGCAGCAATTAGCTATT
TCTGCTGACCCCTCCCGGGGAGCCCGTAGTTGTTGTACTTGTCTTGTCTAATCAGCCGATAATCTTTCCGGTAGGAGCCGTCGTTAATCGATAA

600

→ g r l g g r p r a i n k h e q e q i s r l l l e k g h p r q q l a i
d d w e g a l g r s t n m n r n r l v g y . r k a i l l g s n . l l
r t t g r a p s g d q q t . t g t d . s a i r e r p s s a a i s y y
p r s p p r g r a i l l c s c s c i l r s n s f p w g r c c n a i
l s s q s p a r p r d v f m f l f l n t p . . l f a m r p l l . s n
l v v p l a g e p s . c v h v p v s . d a i l s l g d e a a i l .

ATTTTGGTATTGGCGTATCCACCTTATACAGTACTTCCGGCAAGCAGTATAAAAACGATGATTTTCTTACTTAATTTTATTTTACGTGTGCTCTACCTATATY
700
TAAAAACCATAACCCGATAGGTGGAATATGTCTATGAAAGGCCGTTCGTCATATTTTTTCTTACTTAATTTTATTTTACGTGTGCTCTACCTATATY

↓ end hin ORF
→ i f g i g v s t l y r y f p a s s i k k r m n . n k n h n r m d i t
f l v l a y p p y t d t f r q a v . k n e . i k i k i t t g w i .
f w y w r i h l i q i l s g k q y k k t n e l k . k s q q d g y n
i k p i p t d v k y l y k g a l l i f f r i f . f l f . l l l i s i
n k t n a y g g . v s v k r c a t y f f s h i l i f i v v p h i y
. k q y q r i w r i c i s e p l c y l f v f s n f y f d c c s p y l

CATTTTGTAAATACAGCGTATGGCATAAATAAACCGAAAGGGTATACAAAAAGACAGCATCTAATTAAGAGAGAAAAAATTCACGTATTAACATAT
800
GTAAAAACATTATGTCCGCATACCGTATTTATTTGGCTTTCCCATATGTTTTTCTGCTGATAGATTAATTTTCTCTTTTTAAGTTGCATAATTGTATA

f l . y r r m a . i n r k g i q k r q h l i k k r k n s t y . h i
h f c n t g v w h k . t e r v y k k d s i . l k r e k i q r i n i y
i f v i q a y g i n k p k g y t k k t a s n . k e k k f n v l t y
v n k y y l r i a y i f r f p i c f l c c r i l f l f f e v y . c i
c k q l v p t h c l y v s l t y l f s l m . n f l s f i . r i l m
m k t i c a y p m f l g f p y v f f v a d l . f s f f n l t n v y

Styl
↓
ATAGTGTAAACGCGCTCAGCATAAGGCCATGTTACATCCAGCTATAGACGACATCGCTCAAAACACTACCAGACACAGTATTCACCTGGAAAGGCTTTTT
900
TATCACATTGCGCGAGTGCTATTCGGGATACAATGTAGGTCGATATCTGCTGTAGCGAGTTTTGTGATGGTCTGTGTCTAAGTGGACCTTTCCGAAAAA

. c n a l t i r p m l h p a i d d i a q n t t r h s i h l e r l f
s v t r s r . g l e y i q l . t t s l k t l p d t v f t w k g f l
i v . r a h d k a y v t s s y r r h r s k h y q t q y s p g k a f .
y h l a s v i l g i n c g a i s s m a . f v v l c l i . r s l s k
y l t v r e r y p r h . m w s y v v d s l v s g s v t n v q f p k k
i t y r a . s l a . t v d l . l r c r e f c . w v c y e g p f a k

flagellin gene promoter →
AATCAAAATGTTAGATGTAAGCAATTACGGACAGAAAAATAGTAAAGTTTATGCCTCAAGTGTGATAACCTGGATGACACAGGTAAGCCTGGCATAAC
1000
TTAGTTTACAATCTACATTCGTTAATGCCTGTCTTTTTTATCATTTCAAATACGGAGTTCACAGCTATTGGACCTACTGTGTCCATTGGACCGTATTG

n q n v r c k q l r t e k i v k f m p q v s i t w m t q v s l a . h
i k m l d v s n y g q k k . . s l c l k c r . p g . h r . a w h n
s k c . m . a i t d r k n s k v y a s s v d n l d d t g k p g i t
l . f t l h l c n r v s f i t f n i g . t d i v q i v c t l r a y
i l i n s t l l . p c f f y y l k h r l h r y g p h c l y a q c l
. d f h . i y a i v s l f f l l t . a e l t s l r s s v p l g p m v

—IRR—
ATTGGTTATCAAAAACCTTCCAAAAGGAAATTTTATGGCACAAGTAACTCAACACTAACAGTCTGTGCTGTGACCCAGAATAACCTGAACAAATCCCA
1100
TAACCAATAGTTTTGGAAGGTTTTCTTTTAAATACCGTGTTCATTAGTTGTGATTGTGACACAGCGACGACTGGGTCTTATTGGACTTGTTTAGGT

↓ H2 flagellin structural gene begin ORF
→ w l s k t f q k e n f m a q v i n t n s l s l l t q n n l n k s q
i g y q k p s k r k i l w h k . s t l t v e r c . p r i t . t n p s
l v i k n l p k g k f y g t s n q h . q s v a a d p e . p e q i p
c q n d f v k w f s f k i a c t i l v l l r d s s v w f i r f l d w
m p . . f g e l l f i k h c l y d v s v t q r q q g l i v q v f g
n t i l f r g f p f n . p v l l . c . c d t a a s g s y g s c i g

GTCCGCACTGGGCACCGCTATCGAGCGTCTGTCTTCTGGTCTGCGTTCC
1149
CAGGCGTGACCGTGCGGATAGCTCGCAGACAGAAGACCAGACGCAAGG

H2 ORF
→ s a l g t a i e r l s s g l r s
p h w a p i s s v c l l v c v p
v r t g h r y r a s v f w s a f h
d a s p v a i s r r d e p r r e v
l g c q a g s d l t q r r t q t g
t r v p c r . r e d t k q d a n w

was screened for homology to the *S. typhimurium* DNA using the 678 bp ("680 bp") *BscI/StuI* fragment of pKK1001B as a probe (MacGregor, 1990). One clone which hybridised to the probe was called pDI116, or p16 in this study. Restriction enzyme digestion of the DNA of the clone showed a 0.58 kb *BscI* fragment to be present. Analysis of the chromosomal DNA of *B. pertussis* (Taberman and Tohama strains) by Southern blots showed the 680 bp probe bound to a 0.58 kb *BscI* or *ClaI* fragment, *ClaI* an isoschizomer of *BscI* (MacGregor, 1990). Examination of the DNA of other *Bordetella* species showed binding of this 680 bp probe to a 0.9 kb *BscI* fragment of *B. bronchiseptica* DNA but not to *B. parapertussis* DNA strain NCTC 10520. Chromosomal DNA of *B. avium* strains 4041 and 4049 could not be digested by *BscI* but were digested by *StuI* and the probe hybridised to two fragments, of 3.8 and 4.0 kb.

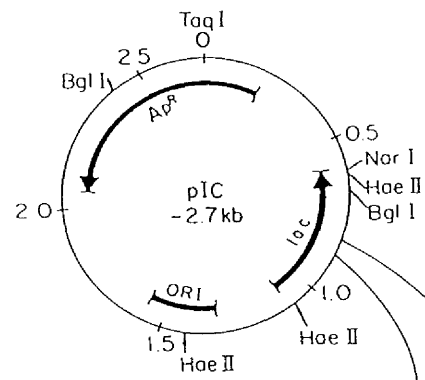
3.2. Restriction enzyme digestion of the DNA of *B. pertussis* cosmid clone, p16, to show the presence of a 0.58 kb *BscI* DNA fragment.

Digestion of the DNA of clone p16 with *BscI* gave fragment sizes that corroborated the map of MacGregor (1990). There is still ambiguity as to the exact order of the *BscI* fragments around the 0.58 kb *BscI* fragment. Accurate sizing was difficult due to it being necessary to overload the DNA on the electrophoresis gel to visualise the 0.58 kb fragment.

3.3. Subcloning the 0.58 kb *BscI* fragment into the plasmid pIC20H.

To produce more of the 0.58 kb DNA in order to prepare a probe and for future plasmid sequencing analysis, the 0.58 kb fragment was subcloned into a smaller vector, plasmid pIC20H (Fig. 3.3). The DNA of pIC20H was digested with *BscI* and dephosphorylated with CIP, while p16 was also digested with *BscI*. Fragments were separated by electrophoresis on LMP agarose (Fig. 3.4) and the relevant DNA bands were cut out. The agarose was melted and diluted with sterile distilled water and the DNA recovered by adsorption onto GeneClean.

Fig. 3.3. Plasmid cloning vector, pIC20H, showing multiple cloning sites
(Marsh *et al.*, 1984).



pIC-2.0H Met Thr Met Ile Thr Pro Ser Leu His Ala Cys Arg Ser Thr Leu Glu Ala Pro Arg Val Pro Ser Ser Asp Ser Ser Ile Ser Arg Ser Arg Ala Arg Glu Ser Leu Ala Leu Arg
 ATG ACC ATG ATT ACC CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCG CGG TAC CCG AGC TCG CAT TCA TCG ATA TCT AGA TCT CGA GCT CCG CAA AGC TTG GGC TCG GCC
 Hind III Sph I Pst I Sal I Xba I BamHI Kpn I Sac I Eco RI Cla I Xba I Kpn I Hru I Hind III
 Sma I Eco RV Bgl II Sac I

The DNA fragments were ligated with DNA ligase prior to transformation of *E. coli* JM83 competent cells. Twelve white and two blue colonies were chosen and purified by streaking to single colonies on selective plates containing ampicillin and X-gal. Plasmid DNA was prepared from all fourteen colonies and digestion of the plasmids with *BscI* showed that this site no longer was available for digestion as the recombinant DNA was not digested. Using the *BscI* site for the insertion appeared to have affected the DNA preventing further digestion, perhaps by altering a base at the recognition sequence. Two *HindIII* sites were present in the pIC20H polylinker flanking the *BscI* site so the plasmids were then digested with *HindIII* (Fig. 3.5). This showed that ten of the plasmids including the two plasmids which had originated from the two blue colonies contained a DNA insert. The blue colour presumably indicated that the insert had not affected the reading frame, allowing functional beta galactosidase alpha-peptide to be produced. A Southern blot of the digested recombinant plasmid DNA showed that each insert hybridised with the 0.58 kb fragment (data not shown), confirming that the inserts represented subclones of the original 0.58 kb.

3.4 Analysis of *B. pertussis* chromosomal DNA and clone p16 to obtain a larger fragment containing the 0.58 kb *BscI* fragment.

The DNA *hin* invertase gene is 573 bp but inclusion of all the region between, and including, the inverted repeats increases this to 995 bp. Therefore the 0.58 kb fragment, obtained by *BscI* digestion of clone p16 fragment, might not have included all the *B. pertussis* DNA homologous to *Hin* DNA. It was therefore decided to see if a fragment larger than and containing the 0.58 kb fragment could be obtained from clone p16. Therefore *B. pertussis* chromosomal DNA (strain Taberman, Table 2.1), cosmid vector pLAFRI (Table 2.3) and cosmid clone p16 (Table 2.3) were digested with various restriction enzymes, Southern blotted and hybridised with the 0.58 kb *BscI* fragment, hybridisation conditions as in 2.7.17.b. It would have been better to use the *Hin* DNA

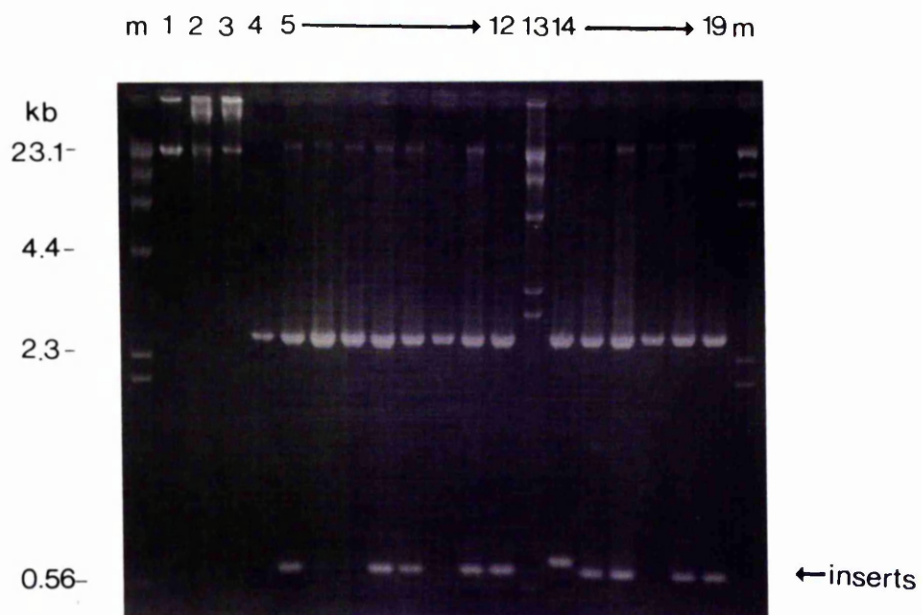
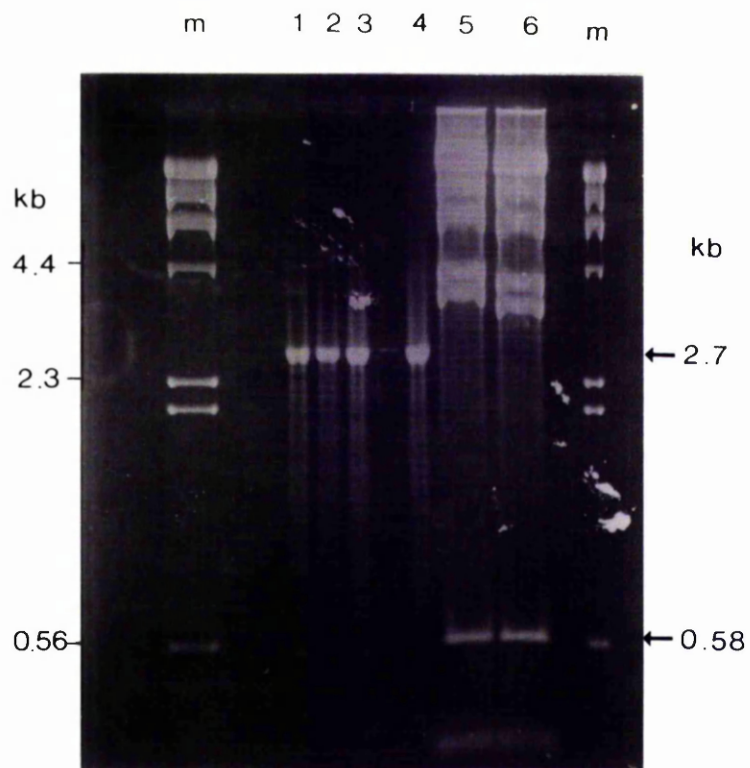
Fig. 3.4. Electrophoresis of *BscI*-digested plasmid vector pIC20H and cosmid recombinant p16 showing the 0.58 kb fragment.

The lambda *HindIII* marker is labelled (m) and sizes are marked on the left of the gel. Electrophoresis in a 1% agarose gel. (Lanes 1-3) *BscI*-digested and dephosphorylated vector pIC20H; (4) *BscI*-digested vector pIC20H; (5,6) *BscI*-digested clone p16. Fragment sizes of interest are marked with arrows to the right of the gel.

Fig. 3.5. *HindIII* digestion of *B. pertussis* chromosomal DNA, vector and recombinant plasmids.

The lambda *HindIII* markers are indicated to the left of the gel. The arrow to the right of the gel indicates insert fragments. Lanes contained DNA prepared from the following plasmids or strains:

(1) Chromosomal *B. pertussis* DNA strain Taberman; (2) cosmid vector pLAFR1; (3) cosmid library clone p16; (4) plasmid vector pIC20H; (5->12, 14->19) subclones pIC20H containing 0.58 kb inserts; all digested with *HindIII*. (13) Plasmid p16 digested with *BscI*.



invertase fragment probe from pKK1001B but there was insufficient DNA and initial attempts to transform the plasmid into *E. coli* to obtain more DNA were unsuccessful. Once a restriction fragment of suitable size e.g. ~2 kb was found DNA sequencing analysis would determine if there was homology to the *Hin* DNA. Digestion of p16 with most restriction enzymes gave fragments which were either too large or produced too many fragments homologous to the probe. However two enzymes, *Pst*I and *Nru*I, gave potentially useful fragments (Fig. 3.6. lanes 4,5,6 and 16,17,18). The main *Nru*I fragment homologous to the probe was smaller in the digest of the chromosomal DNA than in clone p16 (Fig. 3.7. lanes 16,17,18). This may have been due to rearrangements occurring during preparation of the gene library. Digestion with *Pst*I (lanes 4,5,6) yielded more promising results as both the chromosomal and p16 DNA showed binding to the probe with fragments of the same size. However the probe also showed excessive binding to the vector DNA. It was decided therefore to sequence the 0.58 kb fragment in the pIC20H vector to determine if it exhibited homology to the *Hin* DNA.

3.5. Sequencing of the 0.58 kb *Bsc*I DNA fragment subcloned in pIC20H.

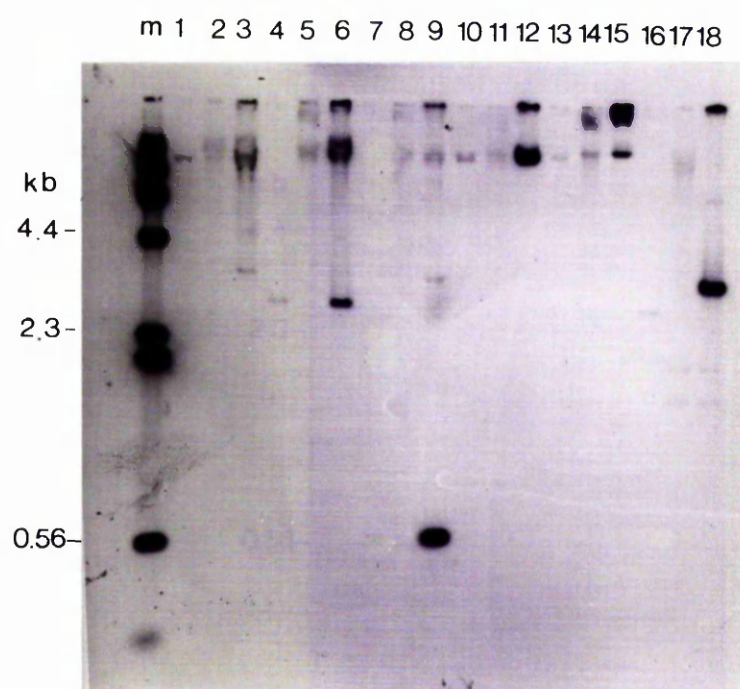
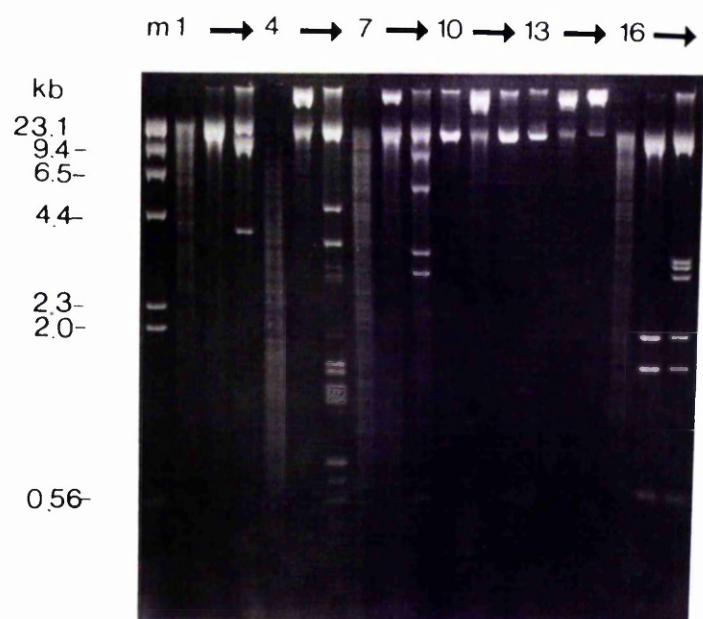
The insert DNA was partially sequenced from one of the pIC20H plasmids containing an 0.58 kb insert, (Lane 11; Fig. 3.5). The sequence obtained using the Sequenase Kit (USB) consisted of 250 bp on one strand and 350 bp on the other strand of DNA. In the plasmid DNA sequencing stops and compressions in the GC rich DNA were found to be a major problem so the insert DNA was recloned into M13 for single strand sequencing. The use of inositol triphosphate (ITP) in the sequencing reaction mix was found to be successful in resolving the problems of stops and compression.

Fig. 3.6. Restriction enzyme digestion of chromosomal DNA from *B. pertussis* strain Taberman, cosmid vector pLAFR1 and *B. pertussis* cosmid library clone p16.

The lambda *Hind*III markers are indicated to the left of the gel. Lanes contained: chromosomal DNA prepared from *B. pertussis* Taberman strain (lanes 1,4,7,10,13,16); plasmid pLAFRI (lanes 2,5,8,11,14); and cosmid clone p16 (lanes 3,6,9,12,15 and 18). The DNA was digested with the following: (lanes 1,2,3) *Eco*RI; (4,5,6) *Pst*I; (7,8,9) *Bsc*I; (10,11,12) *Xba*I; (13,14,15) *Sac*I; (16,17,18) *Nru*I.

Fig. 3.7. Southern blot analysis of *B. pertussis* chromosomal DNA, cosmid vector pLAFR1 and cosmid clone p16 (gel, Fig. 3.6.) hybridised with the 0.58 kb *Bsc*I DNA fragment probe.

The lambda *Hind*III markers (m) sizes are indicated to the left of the blot. Lanes contained DNA as noted in Fig. 3.6.



3.6. Subcloning the 0.58 kb *BscI* fragment into the bacteriophage M13 mp18 and mp19.

The M13 vector DNA has multiple cloning sites with mp18 having a polylinker of opposite orientation to that of mp19. This allows both strands of the insert DNA to be sequenced with one primer. The sequencing of the DNA in the plasmid vector had shown a *SalI* restriction site within the 0.58 kb DNA fragment. This information was used to subclone portions of the 0.58 kb fragment into the M13 vector. Three restriction enzymes were used: *EcoRI*, *SalI* and *HindIII*. The digestion with *EcoRI* and *HindIII* included the whole of the insert. Overlapping sequences aid in confirming the sequence so smaller fragments were also subcloned for sequencing. The insert was cleaved with *EcoRI/SalI* and *SalI/HindIII* and cloned into the relevant sites of the relevant vector. This yielded the following insert fragments which were corroborated by mapping with restriction enzymes (Fig. 3.8). *HindIII* and *EcoRI* digestion of pIC0.58 produced a 54 bp and a 593 bp fragment, and included the whole of the insert. *EcoRI* and *SalI* digestion produced a 42 bp and a 406 bp fragment which included part of the insert. While *HindIII* and *SalI* digestion produced a 21 bp, a 436 bp (*SalI/SalI* fragment) and a 186 bp fragment which included the rest of the insert fragment not covered by the *EcoRI/SalI* restriction digestion. Where sequential digestions required enzymes to be in different buffers; after the first digestion the DNA was precipitated with ethanol, resuspended in sterile distilled water and the second buffer then added. DNA fragments were ligated into dephosphorylated M13 vector DNA and the recombinant DNA transformed into competent *E. coli* TG1. From each set of transformations, 12 plaques were picked for preparation of DNA for sequencing.

Fig. 3.8. Schematic representation of restriction digest products of the vector pIC20H containing the 0.58 kb insert digested with *Eco*RI, *Sal*I and *Hind*III.

Order of polylinker sites present in pIC20H:-

HindIII - - *SalI* - - *EcoRI* - - *ClaI* - - *HindIII*

0.58 kb

SalI

The ~0.58 kb fragment (containing a *SalI* site) was inserted into the *ClaI/BscI* site of pIC20H.

***HindIII* and *EcoRI* double digestion products of the vector pIC20H containing 0.58 kb DNA fragment.**

HindIII | 54 bp | *EcoRI* *EcoRI* | 6bp + 560 bp + 27 bp = 593 bp | *HindIII*

***EcoRI* and *SalI* double digestion products of the vector pIC20H containing 0.58 kb DNA fragment.**

SalI | 42 bp | *EcoRI* *EcoRI* | 6 + 400 = 406 bp | *SalI*

***HindIII* and *SalI* double digestion products of the vector pIC20H containing 0.58 kb DNA fragment.**

HindIII | 21 bp | *SalI* *SalI* | 36 + 400 = 436 bp | *SalI*

SalI | 160 + 27 bp = 186 bp | *HindIII*.

The fragment with the *SalI-SalI* ends does not insert into the *SalI/HindIII* digested vector.

3.7. DNA sequence of the 0.58 kb *BscI* DNA fragment.

The full sequence of the ~0.58 kb fragment is shown in Fig. 3.9.

When clone p16 was analysed with various restriction enzymes for a larger fragment which included the 0.58 kb fragment (Section 3.4) enzymes with sites within the 0.58 kb sequence were also found (data not shown). These restriction sites have again been shown to be present on sequencing the 0.58 kb fragment confirming that this was the same insert that was present in p16. The restriction sites are *SphI*, *EcoRV* and *SalI*.

Both DNA strands of the ~0.58 kb fragment were compared with the DNA sequence of the *S. typhimurium* Hin invertase DNA sequence (Silverman *et al.* 1981, Fig 3.2.) using the Align programme (DNASTAR). No significant homology was noted. The DNA sequence was then compared to DNA sequences in GenBank and EMBL using the programme Nucscan again no significant homology was noted. When the sequence was translated it resulted in three reading frames for each DNA strand, but only one reading frame had an open reading frame throughout its whole length. The open reading frame was compared against the known amino acid sequences in NBRF using the Proscan programme. No homology to any particular sequence was apparent although there were small regions of homology to insertion sequence and transposon transposases. The alignment of the sequence with the greatest homology is shown in Fig. 3.10. The area of greatest homology (shown between vertical lines in Fig. 3.10.) was 29.4% identity within 34 amino acids. Underneath is information on the sequences which the Proscan analysis of the ORF (191 amino acids) indicated had some homology to other amino acid sequences.

Fig. 3.9. DNA sequence of the *B. pertussis* ~0.58 kb *Cla*I fragment.

The predicted amino acid sequences and stop codons (.) in all three reading frames for both strands are shown and the restriction sites for *Sph*I, *Eco*RV and *Sal*I.

E
C
R
V



TGCCCTCGGCAGCGCCCTTCGCACGGGCAAACTCCCGGATATCCGGCCGGCGACGAACGTGTTGCCCTCGCACGCCAGCACACCAGCCCGCACTGCGGGG

100

ACGGAGCCGTGCGGGGAAGCGTGCGCGTTTGGGGCCTATAGCGCGGCCGCTGCTTGCAACAGGGAGCGTGCGGTCTGGTGGCGGGCGTGACGCCCC

c l g s a l r t r k l p d i r a g d e r v a l a r q h h r p h c g v
a s a a p f a r a n s r i s a p a t n v l p s h a s t t a r t a g
p r q r p s h a q t p g y p r r r r t c c p r t p a p p p a l r g
r p l a r r v r l s g s i r a p s s r t a r a r w c w r g c q p
a e a a g k a r a f e r i d a g a v f t n g e c a l v v a r v a p
g r c r g e c a c v g p y g r r r r v h q g r v g a g g g a s r p

S
A
L
1



TCCGCTTCCGCCTCGCGCAGCGCCGAGCACAGGCCGGCACGTACCGTATCCGACAGTGC GTTGATCGGCGGATTGTCGACGCTCAGGACGGCTACGTTCC

200

AGGCGAAGGCGGAGCGCGTGCGCGGCTCGTGTCCGGCCGTGCATGGCATAGGCTGTACGCAACTAGCCGCCCTAACAGCTGCGAGTCTGCGGATGCAAGG

r f r l a q r r a q a g t y r i r q c v d r r i v d a q d g y v p
s a s a s r s a e h r p a r t v s d s a l i g g l s t l r t a t f r
p l p p r a a p s t g r h v p y p t v r . s a d c r r s g r l r s
t r k r r a c r r a c a p v y r i r c h t s r r i t s a . s p . t g
d a e a e r l a s c l g a r v t d s l a n i p p n d v s l v a v n
g s g g r a a g l v p r c t g y g v t r q d a s q r r e p r s r e

GATGCCGGGCAACGCTCACCACCGGCTTGATGTGTTGGAAGTCCGGGTCGTATTCTCGACCTGCCTCCAGCTGGTTGGAAATGCTACGTGTAGGGAAA

300

CTACGGCCCCGTTGCGAGTGGTGGCCGAACACACAAGCTTCAGGCCCAAGCAGTAAGAGCTGGACGGAGGTCGACCAACCTTTACGATGCACATCCCTTT

m p g n a h h r l d v f e v r v r h s r p a s s w l e m l r v g k
c r a t l t t g l m c s k s g f v i l d l p p a g w k c y v . g n
d a g q r s i p p a . c v r s p g s s f s t c l q l v g n a t c r e i
i g p l a . w r s s t n s t r t r . e r g a e l q n s i s r t p f
r h r a v s v v p k i h e f d p n t m r s r g g a p q f h . t y p f
s a p c r e g g a q h t r l g p e d n e v q r w s t p f a v h l s

TCGCCACGTTGGCCTGGGCAACGTGGACAGCATGAAATCTACACGCCTAACCTCGCCGCAATCAGGCCAAGTATTCGCATATTCGAATTATTCATTGATA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 400
AGCGGTGCAACCGGACCCGTTGCACCTGTCGTACTTTAGATGTGCGGATTGGAGCGCGGTTAGTCCGGTTCATAAGCGTATAAGCTTAATAAGTAAGTAT

s p r w p g q r g q h e i y t p n l a a i r p s i r i f e l f i h k
r h v g l g n v d s m k s t r l t s p q s g q v f a y s n y s f i
a t l a w a t w t a . n l h a . p r r n q a k y s h i r i i h s .
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
d g r q g p c r p c c s i . v g l r a a i l g l i r m n s n n m .
r w t p r p l t s l m f d v r r v e g c d p w t n a y e f . e n m
i a v n a q a v h v a h f r c a . g r r l . a l y e c i r i i . e y

AGCATGAATACTGCGCTCAGCCGTTGATTGATGTTTCGCGCCCTGGTACTCTTCGTTCCATCAGAACTGGCGCATACCTCGAAGCCGGTCGCATACAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 500
TCGTACTTATGACGCGAGTCGGCAACTAAGCTACAAGCGCCGGACCCATGAGAAGCAAGGTAGTCTTGACCGCGTATGGAGCTTCGGCCAGCGTATGTGC

h e y c a q p l i r c s r p g y s s f h q n w r i p r s r s h t r
s m n t a l s r . f d v r g l g t l r s i r t g a y l e a g r i h g
a . i l r s a v d s m f a a w v l f v p s e l a h t s k p v a y t
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
l c s y q a . g n i r h e r g p y e e n w . f q r m g r l r d c v r
l m f v a s l r q n s t r p r p v r r e m l v p a y r s a p r m c
a h i s r e a t s e i n a a q t s k t g d s s a c v e f g t a y v

S
P
H
1
↓

GAGACAACACGCATGCCGCTGGCGAATCATGAAGTTTCACCATCACGGCCCCGCACCGCGGACGCGGCCGAATCG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 575
CTCTGTTGTGCGTACGGCGACCGCTTAGTACTTCAAAGTGGTAGTGCCGGGGCGTGGCGCCTGCGCCGGCTTAGC

r q h a c r w r i m k f h h h g p a p r t r p n r
d n t h a a g e s . s f t i t a p h r g r g r i v
e t t r m p l a n h e v s p s r p r t a d a a e s
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
l c c a h r q r i m f n . w . p g a g r v r g f r
p s l v c a a p s d h l k v m v a g c r p r p r i a
s v v r m g s a f . s t e g d r g r v a s a a s d g

Fig. 3.10. Comparison of ~0.58 kb DNA-translated largest open reading frame to insertion sequence IS3411 transposase.

(1) ~0.58 kb DNA-translated largest open reading frame (ORF) from 1 to 191 amino acids compared to (2) insertion sequence IS3411 transposase 1 to 240 amino acids, as aligned by the Proscan programme, DNASTAR.

Where an amino acid is written between the two sequences, it is common to both sequences. Highly homologous and less homologous amino acids are represented by double and single dots, respectively. The area of greatest homology is shown between the vertical lines marked on the sequences.

					10v	20v
(1)					CLGSALRTRKLPDIRAGDERVAL	
					G. :RT .. A::RV.	
(2)	NHKVYGVRKVWRQLLREGIRVARCTVARLMVVMGLAGVLRGKKVRTTISRKAVAAGHRVNR					
	70^	80^	90^	100^	110^	120^

	30v	40v	50v	60v	70v	80v	
(1)	ARQHHRPHCGVRFR	LAQRR	AQAGTYRIRQC	VDRRIVDAQ	DGYVPM	PGNAHHR	LDVFEVRVR
	:RP:	:: :G :	:D :	V: : ::	LD.:E ::		
(2)	QFVAERPDQLWVAD	FTYVSTWRG	FVYVAFI	IDVFAGYIV	GWRVSS	SMETTFV	LDAL
	130^	140^	150^	160^	170^	180^	

(1) 90v 100v 110v 120v 130v 140v
HSRPASSWLEMLRVGKSPRWPGQRGQHEIYTPNLAAIRPSIRIFELFIHKHEYCAQPLIRC
.RP:: ::::V . RWP::G :. : :::: . : :.
(2) TRRPPARSITVIKVLSMYRWPTHSLRKP DYWHQQEVQATRM TTRWRRA SMVFTKRR
190^ 200^ 210^ 220^ 230^ 240^

Homologous sequence	Amount of ORF (191 aa) showing some homology	Most homologous region (N ^o aa) within 191 aa sequence.
---------------------	--	--

<i>E. coli</i> transposase IS3411	139 aa	76 to 105
<i>E. coli</i> transposase Tn3	189 aa	141 to 157
<i>Agrobacterium tumefaciens</i> IS136	147 aa	87 to 109
<i>E. coli</i> transposase Tn21	121 aa	3 to 25
<i>E. coli</i> transposase Tn 2501	127 aa	6 to 25
<i>E. coli</i> transposase Tn 3926	121 aa	6 to 25

The areas of greatest homology, although interesting, are small and thus probably have limited significance. As no sequence homology of the 0.58 kb fragment could be found to the DNA invertase of *S. typhimurium* or surrounding sequences, some doubt was cast on the conclusions of the original hybridisation results of MacGregor (1990). It was decided therefore to reexamine the hybridisation pattern of the *Hin* DNA invertase probe to *Bordetella* DNA.

3.8. Southern hybridisation of *Bordetella* DNA and pKK1001B with the 0.58 kb fragment DNA probe.

Attempts to hybridise the 0.58 kb *BscI* fragment probe to the pKK1001B 680 bp *BscI*/*StuI* fragment or indeed any of pKK1001B had been unsuccessful. The stringency of the conditions were therefore reduced. The conditions of Foxall *et al.* (1990) were investigated and the membrane changed from Hybond-N to Zeta-Probe as used by MacGregor (1990). None of these changes enabled the hybridisation to occur. Various *Bordetella* species were examined (Table 2.4) and chromosomal DNA prepared. The results of electrophoresis of

*Cla*I-digested *Bordetella* DNA followed by Southern blotting of the gel and hybridisation with the 0.58 kb probe, are shown in Fig. 3.11. The probe did not bind to pKK1001B (lane 1), *S. typhimurium* DNA (lane 2), *E. coli* DNA (lane 3) or the negative control *Peptostreptococcus magnus* W1384 (lane 4). The 0.58 kb probe did, however, bind to a 0.58 kb fragment in various strains of *B. pertussis* DNA (lanes 5,6,7,8,10,12) and hybridised to Arg 41 DNA although this was an incomplete digest (11). Hybridisation to 0.58 kb fragments in *Cla*I-digested DNA from *B. pertussis* Taberman and Tohama strains was also shown by MacGregor (1990) when using the pKK1001B *Cla*I-*Stu*I 680 bp fragment as a probe. In contrast to his results, the 0.58 kb probe in this study also hybridised to a 0.58 kb fragment in *B. parapertussis* (lane 13) but not to *B. bronchiseptica* (lane 9) or *B. avium* (lane 14) DNA. The positive controls, p16 and subclone pIC20H containing 0.58 kb insert (lanes 15,16,17) did hybridise.

3.9. Discussion.

Previous work had shown the hybridisation of a *S. typhimurium* DNA probe, which included *Hin* invertase DNA, to a *B. pertussis* cosmid library clone, p16, and a 0.58 kb *Cla*I fragment in *B. pertussis* chromosomal DNA (MacGregor, 1990). This appeared to be consistent as the clone also contained a 0.58 kb *Cla*I fragment. Analysis of the cosmid clone, p16, in this study confirmed the presence of the 0.58 kb *Cla*I fragment which when used as a probe hybridised to a 0.58 kb fragment in *B. pertussis* chromosomal DNA. However, the converse using the 0.58 kb fragment of *B. pertussis* DNA to probe the *S. typhimurium* DNA did not show any evidence of hybridisation. When the photograph of the analysis of the cosmid library by MacGregor (1990) was re-examined, several clones hybridised suggesting that the conditions were of insufficient stringency. Sequence analysis of the 0.58 kb fragment showed no clear homology to known sequences and appeared to be *Bordetella* DNA with only limited homology to *S. typhimurium* DNA. This indicates that the hybridisation procedure used by MacGregor (1990)

should be repeated. It was essential to transform pKK1001B into *E. coli* in order to prepare enough plasmid DNA for use as a probe.

Fig. 3.11. Southern blot analysis of restriction enzyme-digested chromosomal DNA of *Bordetella* species hybridised with the 0.58 kb *Cla*I *B. pertussis* DNA fragment probe.

The lambda *Hind*III markers are indicated to the left of the blot. The arrow to the right of the blot indicates the 0.58 kb fragment. Lanes contained *Cla*I-digested DNA (except where indicated) prepared from the following plasmids or strains:

(1) pKK1001B (2) *S. typhimurium* LT2 (digested with *Bsc*I and *Stu*I); (3) *E. coli*; (4) *Peptostreptococcus magnus* W1384;

B. pertussis: (5) Taberman; (6) Tohama; (7) Wellcome 28; (8) L84 I; (10) Arg 40; (11) Arg 41; (12) L84 IV; (9) *B. bronchiseptica* 452 ; (13) *B. parapertussis*: 5952; (14) *B. avium* 4148; (15) Clone p16 from *E.coli* (*dam*⁺); (16) Clone p16 from *E. coli* (*dam*⁻).

(17) pIC20H containing 0.58 kb insert (digested with *Hind*III).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 - 15 16 17

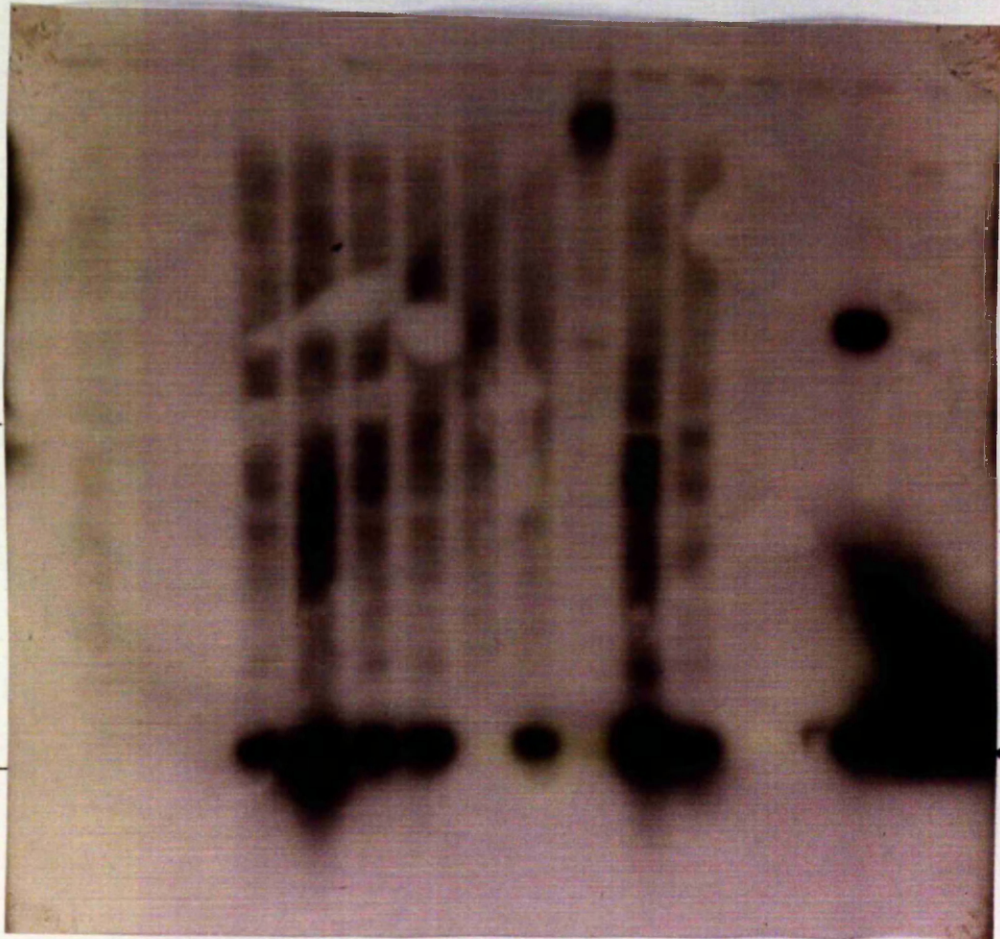
kb

4.4 -

2.3 -

0.56 -

← 0.58kb



CHAPTER 4

IDENTIFICATION OF *BORDETELLA* DNA FRAGMENTS WHICH HYBRIDISE TO HIN DNA AND DNA INVERTASE TARGET SEQUENCES.

4.1. Introduction.

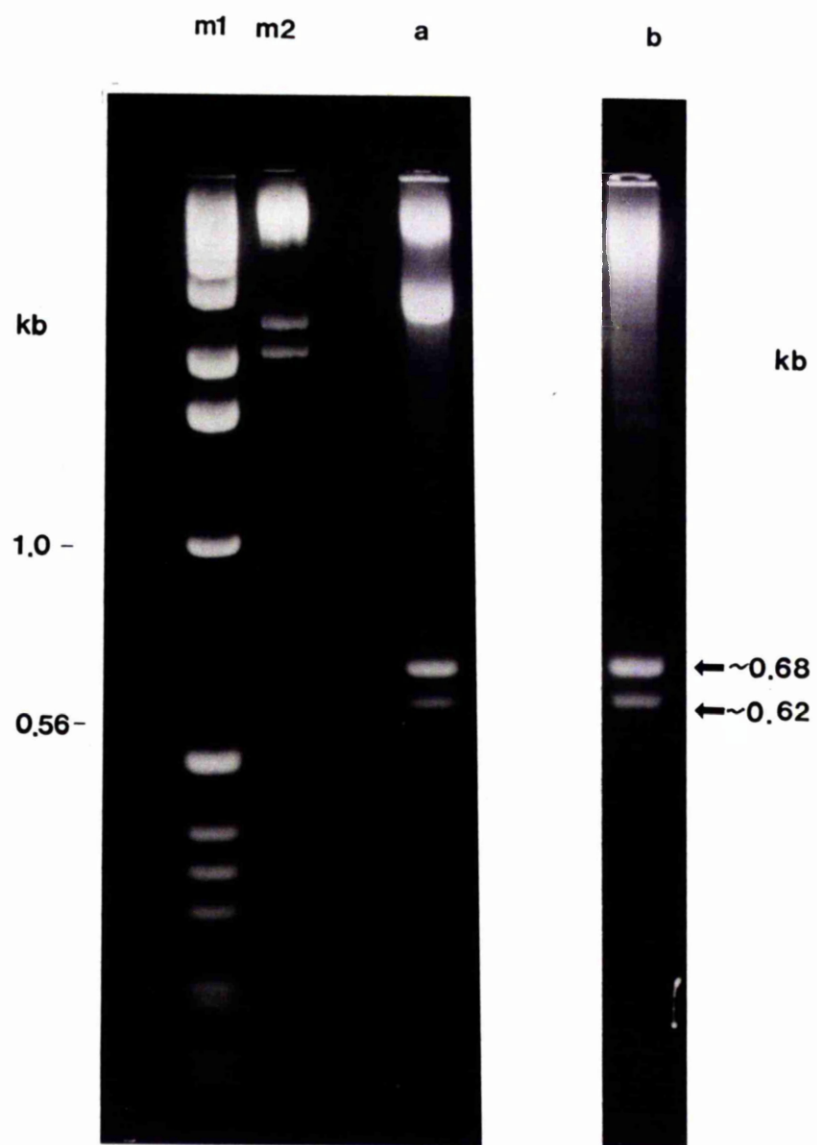
The pKK1001B plasmid consists of a pBR322 vector containing an insert of 8.8 kb of *S. typhimurium* DNA. Restriction enzyme digestion of pKK1001B with *BscI* and *StuI* produces a 680 bp fragment (Fig. 4.1) which includes *Hin* DNA. As there was insufficient pKK1001B DNA available to use as a probe, it was necessary to transform the DNA into *E. coli*, preferably a *dam*⁻ *E. coli* strain, so that it could be digested by restriction enzymes sensitive to Dam-methylated DNA e.g. *BscI*. The DNA of *B. pertussis* clone p16 which had been previously shown to hybridise to the ~680 bp pKK1001B fragment (MacGregor, 1990) was also transformed into the *dam*⁻ *E. coli* cells.

MacGregor (1990) showed homology between the 680 bp probe, containing the *Hin* DNA, and *B. pertussis* *ClaI*-digested chromosomal DNA. This Southern hybridisation was repeated to determine if the same result could be obtained. The *Bordetella* chromosomal DNA chosen for this study was prepared from strains chosen for their different serotypes. AGG 1 was always present in wild-type phase I isolates, whilst AGG 2 and 3 could be lost or regained independently at a high rate (10^{-3} - 10^{-4}) per generation. This rate might suggest that some form of DNA rearrangement, perhaps by a DNA invertase, might be involved in the serotype variation. This could possibly be detected by variation in profiles of DNA hybridisation of these serotype variant strains using the *Hin* probe. *Bordetella* strains which varied in their mode and phase were also examined for differences in hybridisation to the probe. In this study hybridisation of the *ClaI*-digested *Bordetella* chromosomal DNA was repeated and further restriction enzyme digestions were also examined. The *Bordetella* DNA was also hybridised with the 620 bp fragment of *S. typhimurium* produced by pKK1001B digested with *BscI* and *StuI* to see if it too showed homology. If a fragment homologous to DNA invertase were present in the *Bordetella* DNA this could be confirmed by the presence of specific inverted repeat sequences common to invertible DNA. Therefore hybridisation using oligonucleotides constructed

Fig. 4.1. The plasmid pKK1001B from *E. coli* a) (*dam*⁻) and b) (*dam*⁺) strains digested with *BscI* and *StuI*.

The mol. wt. markers: kilobase, Gibco-BRL (m1) and *HindIII* digested lambda (m2) are shown along with their sizes.

(a) Plasmid pKK1001B from an *E. coli* (*dam*⁻) strain digested with *BscI* and *StuI* and the fragments separated by electrophoresis on a 1.5% agarose gel. (b) Plasmid pKK1001B from an *E. coli* (*dam*⁺) strain digested with *BscI* and *StuI* and the fragments separated by electrophoresis on a 1.5% agarose gel.



from these sequences were also used.

4.2. Transformation of pKK1001B and p16 into a *dam*⁺ *E. coli* strain.

Competent *E. coli* DH5 α cells, 100 μ l, were transformed, by mixing with 1 μ l of 1 μ g pKK1001B DNA (Section 2.6.2.). Selected transformant colonies were streaked on agar plates containing ampicillin and the plasmid DNA prepared (Section 2.7.2).

This was digested with *Bsc*I and *Stu*I and the fragments separated by electrophoresis on a 1.5 % agarose gel to separate of \sim 680 and \sim 620 bp fragments. Although both fragments are of similar size, the \sim 680 bp fragment appeared to be in a greater molar ratio (Fig. 4.1.a). This could have been due to inefficient enzyme digestion because of methylation occurring, as the plasmid DNA was from a *dam*⁺ strain. To investigate this pKK1001B was also transformed into a *dam*⁻ strain.

4.3. Transformation of pKK1001B and p16 into a *dam*⁻ *E. coli* strain.

Competent cells of the *E. coli dam*⁻ strain GM242, were transformed with pKK1001B. Digestion of pKK1001B with *Bsc*I and *Stu*I now produced two distinct large fragments as opposed to a doublet (Section 3.1.) but there was no change in appearance of the \sim 680 or \sim 620 bp fragments (Fig. 4.1.b). Therefore methylation was not producing the difference in intensity of these fragments.

The *B. pertussis* clone, p16, which MacGregor (1990) found to have homology to the 680 bp probe was also transformed into the *dam*⁻ *E. coli* strain. Digestion of the demethylated p16 DNA with *Cla*I showed many more bands than with p16 when produced from the *dam*⁺ strain. The appearance of the 0.58 kb *Cla*I fragment from the p16 digest, however, was not affected by the change in methylation state of DNA. Both methylated and non-methylated p16 DNA were used in Southern blots.

4.4. Hybridisation of *Cla*I-digested *Bordetella* DNA with 680 bp probe.

Digestion with *Bsc*I and *Stu*I of pKK1001B isolated from the *dam*⁻ *E. coli* strain produced several fragments including the 680 bp fragment consisting of 526 of the 573 bp of the *hin* gene (DNA sequence Fig. 3.2) which was to be used as a probe. All the fragments were separated by electrophoresis on LMP agarose and the 680 bp DNA band cut out. The agarose was melted and diluted with sterile distilled water as in Section 2.7.16.a. and used for radiolabelling. The *Bordetella* chromosomal DNA was digested with *Cla*I and alkaline-blotted onto Zeta-Probe membrane. Hybridisation conditions (Section 2.7.17.a.) were the same as those used by MacGregor (1990), where this probe hybridised to a 0.58 kb *Cla*I fragment in *B. pertussis* DNA from strains Taberman and Tohama, after a long autoradiographic exposure (two weeks).

In this study the plasmid pKK1001B DNA and *S. typhimurium* DNA were digested with *Bsc*I and *Stu*I while the rest of the DNA was digested with *Cla*I alone. Hybridisation of the 680 bp probe to the blot is shown in Fig. 4.2. The probe hybridised to the 620 bp and the 680 bp fragments in the pKK1001B digest (not shown in figure as the high degree of hybridisation gave rise to a too intense an autoradiographic exposure). The probe also hybridised strongly to *S. typhimurium* DNA in the ~620 bp to ~680 bp region and also to fragments of 1.3 kb and 2.1 kb (lane 1). Hybridisation to *E. coli* showed one main band of 4 kb (lane 2). For *B. pertussis* phase I strains: Taberman, Tohama, Wellcome 28, L84 (lanes 4-7), Arg 40, Arg 41 (lanes 9-10); *B. pertussis* L84 phase IV (lane 11); and *B. avium* 4148 (lane 13), the probe hybridised to a ~0.96 kb fragment and for all these strains except *B. avium* there was weaker hybridisation to a smaller fragment of 0.58 kb (seen more clearly when the film was over-exposed for 14 days). In contrast, *B. bronchiseptica* 452 and *B. parapertussis* 5952 showed fragments hybridising at >10 kb (lanes 8 and 12 respectively). DNA from plasmid p16 from *E. coli* (*dam*⁺) and (*dam*⁻) strains, and subclone pIC20H

Fig. 4.2. Southern blot of *Cla*I-digested *Bordetella* chromosomal DNA hybridised with the ~680 bp fragment.

The 680 bp fragment probe was produced from pKK1001B digested with *Bsc*I and *Stu*I. The mol. wt. (kilobase) marker (Gibco-BRL) is indicated to the left of the blot. The arrows to the right of the blot indicate fragments of interest. Lanes contained *Cla*I-digested DNA (except lane 16) prepared from the following strains: (1) *S. typhimurium* LT2 (digested with *Bsc*I and *Stu*I). (2) *E. coli*; (3) *P. magnus* W1384; *B. pertussis*: (4) Taberman; (5) Tohama; (6) Wellcome 28; (7) L84 I; (9) Arg 40 (10) Arg 41; (11) L84 IV; (8) *B. bronchiseptica* 452; (12) *B. parapertussis* 5952; (13) *B. avium* 4148; (14) Clone p16 from *E. coli* (*dam*⁺); (15) Clone p16 from *E. coli* (*dam*⁻). (16) pIC20H containing 0.58 kb insert (digested with *Hind*III).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



containing 0.58 kb insert (Section 3.3) hybridised at their ~0.58 kb inserts. There was also some weak hybridisation to the DNA of the vectors, although this was possibly non-specific hybridisation due to the low stringency conditions (lanes 14, 15 and 16). The hybridisation with the 680 bp probe to the *Bordetella* DNA showed no difference between various *B. pertussis* serotypes, modes or phases.

4.5. Hybridisation of *BscI*-digested *Bordetella* DNA with the 680 bp probe.

Several DNA preparations from other *Bordetella* strains, including phase variants were investigated. These included *B. pertussis* strains : Tohama, DCH2, Taberman, L84 phases I and IV, 134 phases I and IV, 30042 phases I and IV, 364 phase IV, 11615 phase IV all grown under X-mode conditions and Taberman grown under X and C mode conditions (see Section 1.7.2.). Other *Bordetella* strains tested included *B. parapertussis* strain 5952, several *B. bronchiseptica* strains: A51 phases I and III, 276 phases I and III, FW5 phases I and III and strain 452, and two *B. avium* strains, 4148 and 4480. The chromosomal DNA was digested with *BscI*, Southern blotted and hybridised with the 680 bp probe.

The 680 bp probe bound to a ~0.96 kb *BscI* fragment in all *B. pertussis* strains irrespective of mode or phase. For *B. parapertussis* and *B. bronchiseptica* strains, the probe bound to a band of >10 kb. This could possibly be explained by the DNA not being fully digested, but increasing the enzyme/DNA proportions did not change the result. Two *B. avium* strains were investigated but there was not enough DNA for hybridisation. These results were essentially similar to those obtained with *ClaI*-digested DNA.

EcoRI-digests of the chromosomal DNA were also investigated. Only DNA from *S. typhimurium* and *B. pertussis* strains Taberman (X-mode), Tohama and Arg 40 digested to completion. Incomplete or no *EcoRI* digestion was shown especially by the *B. parapertussis*, *B. bronchiseptica* and *B. avium* strains (data not shown). It is possible that these *Bordetella* species show some methylation at the *EcoRI* restriction site.

4.6. Hybridisation of restriction enzyme digested *B. pertussis* Taberman X-mode DNA, with the 680 bp probe.

Enzymes *SalI*, *MaeI*, *EcoRI*, *XhoI*, *SmaI*, *SacI* digested the DNA to completion whereas *HindIII* showed no digestion. Hybridisation to the digested DNA was to high molecular weight fragments except for *SalI* digestion where a 1.6 kb fragment was noted and *SmaI* digestion where a 2.6 kb fragment was noted (Fig. 4.3). The hybridisation to the > 10 kb *EcoRI* fragment confirmed previous reports of the hybridisation of the *Hin* DNA region probe to a ~15 kb *EcoRI* *B. pertussis* fragment (Foxall *et al.*, 1990).

4.7. Hybridisation of *ClaI*-digested *Bordetella* DNA with the 620 bp probe.

The 680 bp fragment produced by digestion of the plasmid pKK1001B with *BscI* and *StuI* contains the majority of the *hin* gene. As there are no *BscI/ClaI* or *StuI* sites in the vector, the other restriction site producing the 620 bp fragment must also be in the insert. The 620 bp fragment is therefore probably produced by the presence of another *BscI* or *StuI* site on one side of the 680 bp fragment. Restriction digestion of pKK1001B followed by electrophoresis on a 2 % agarose gel resulted in the 680 bp and 620 bp fragments being well separated. It was of interest to discover if the 620 bp fragment also showed homology to the *B. pertussis* DNA. A separate Southern blot was prepared using the same DNA samples as used in the previous hybridisations (Section 4.5.) but this time hybridised with the 620 bp probe. The probe was radiolabelled as in Section 2.7.16.a.

Hybridisation of the fragment (Fig. 4.4.) to the control DNA, pKK1001B, showed binding to both the 620 bp and the 680 bp fragments (hybridisation too strong to be shown in Fig.). For the *S. typhimurium* DNA there was strong hybridisation in the region ~620-680 bp, to ~1.35 kb fragments and less strongly to 1.85 kb, 2.5 kb and 4 kb fragments (lane 1). In *E. coli* DNA there was

Fig. 4.3. Southern blot of restriction enzyme-digested *B. pertussis* chromosomal DNA hybridised with the ~680 bp fragment.

The mol. wt. (kilobase) marker (Gibco-BRL) is indicated on the left of the blot. The arrows on the right of the blot indicate fragments of interest (see text). Lanes contained *B. pertussis* Taberman DNA (1) undigested and digested with the following enzymes: (2) *Hind*III; (3) *Sal*I; (4) *Mae*I; (5) *Eco*RI; (6) *Xho*I; (7) *Sma*I and (8) *Sac*I.

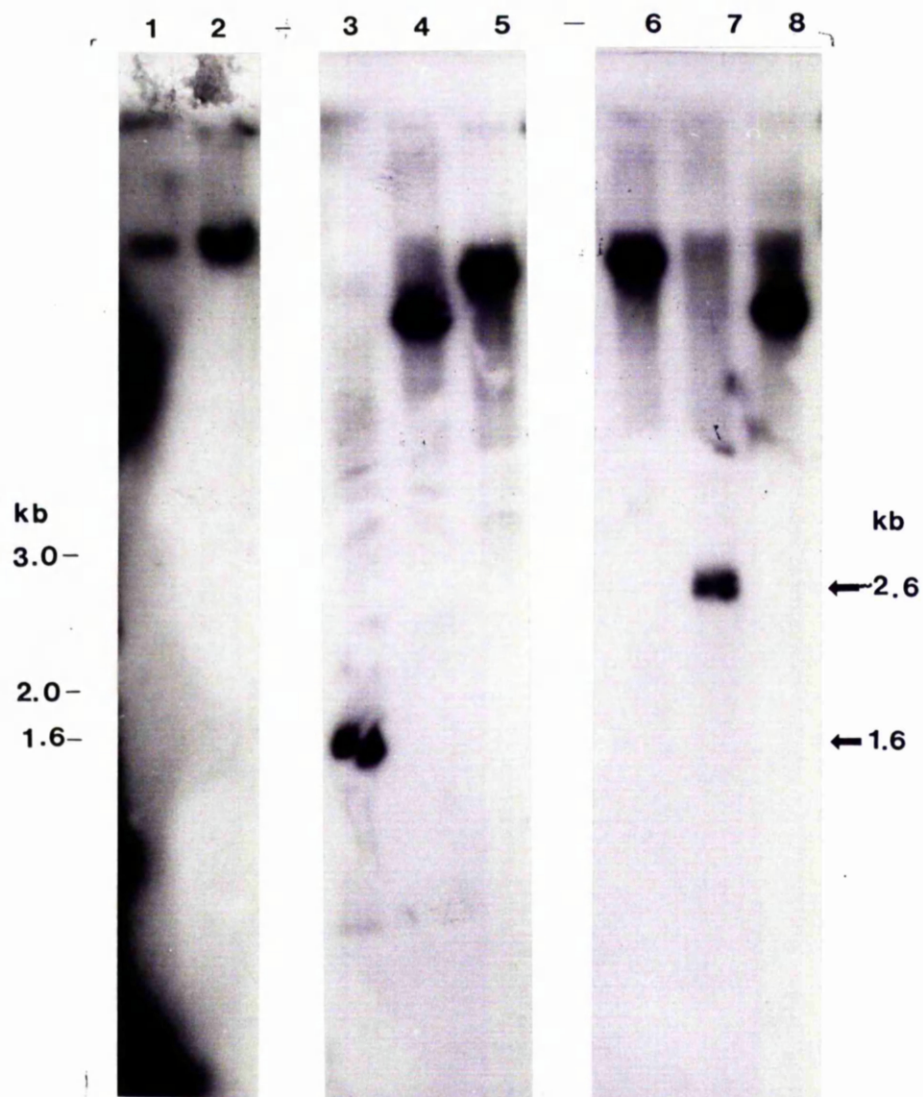
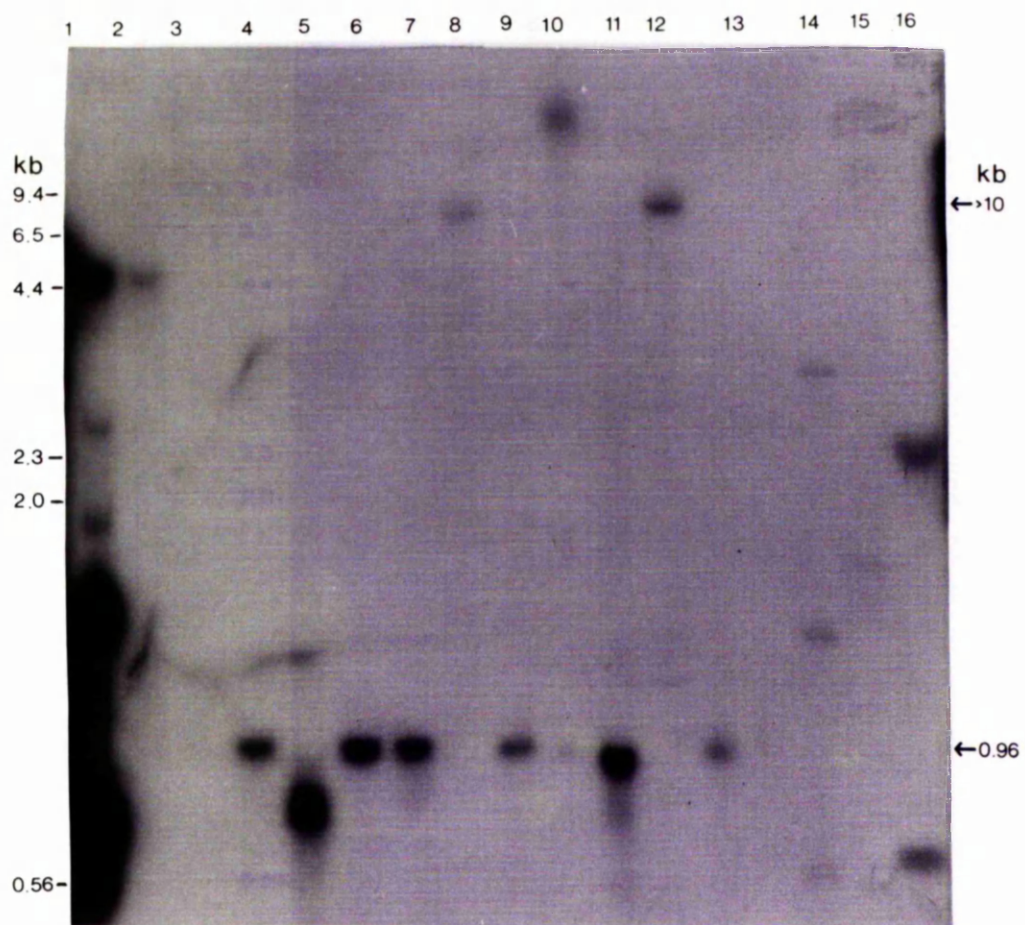


Fig. 4.4. Southern blot of *Cla*I-digested *Bordetella* chromosomal DNA hybridised with the ~620 bp fragment.

The *Bsc*I-*Stu*I 620 bp fragment from pKK1001B. The lambda *Hind*III marker is indicated to the left of the blot. The arrows to the right of the blots indicate fragments of interest (see text). Lanes contained *Cla*I-digested DNA (except where stated) prepared from the following strains: (1) *S. typhimurium* LT2 (digested with *Bsc*I and *Stu*I). (2) *E. coli*; (3) *P. magnus* W1384; *B. pertussis*: (4) Taberman; (5) Tohama; (6) Wellcome 28; (7) L84 I; (9) Arg 40 (10) Arg41 IV; (11) L84 IV; (8) *B. bronchiseptica* 452; (12) *B. parapertussis* 5952; (13) *B. avium* 4148; (14) Clone p16 from *E. coli* (*dam*⁺); (15) Clone p16 from *E. coli* (*dam*⁻). (16) pIC20H containing 0.58 kb insert (digested with *Hind*III).



hybridisation to a single fragment of 4 kb (lane 2). The *B. pertussis* (lanes 4,6,7, 9-11) and *B. avium* DNA (lane 13) showed hybridisation to a ~0.96 kb fragment. Strain Tohama appeared to produce a smaller fragment but this may have been due to overloading of the sample affecting the movement of the DNA on electrophoresis (lane 5). DNA from *B. bronchiseptica* and *B. parapertussis* strains showed hybridisation to a fragment > 10 kb (lanes 8 and 12 respectively). Unfortunately DNA from *B. pertussis* strain Arg 41 was incompletely digested (lane 10). Prolonged exposure of the blot also showed hybridisation to a ~0.56 kb band in *B. pertussis* and *B. parapertussis* strains. There was also hybridisation to the 0.58 kb *Cla*I insert DNA of the p16 and pIC20H subclones containing 0.58 kb inserts and it was also slightly apparent in the vector DNA (lanes 14, 15 and 16).

Curiously, the hybridisation shown by the 620 bp fragment was very similar to, if not the same as, that of the 680 bp fragment (Fig. 4.2). Care was taken on producing the probes that the fragments were well separated on electrophoresis. Thus it is unlikely that one fragment was contaminated with the other. The hybridisation was repeated and the 620 bp probe again bound to the same fragments as the 680 bp probe (data not shown).

The similarity between the 680 bp and the 620 bp hybridisation patterns would suggest that the probes were in some way homologous.

4.8. Search for the presence of DNA homologous to DNA invertase target sequences.

If a DNA invertase were present in *Bordetella* it would act on inverted repeats. Known invertase proteins function on invertible DNA characterised by the presence of conserved inverted repeat sequences. Conserved sequences in the recombination sites of the Hin-related inversion systems are shown in Fig. 4.5. To determine the presence of related inverted repeats, various mixed sequence oligonucleotides were synthesised and used as probes against

Fig. 4.5. Recombination sites of the Hin-related inversion systems.

The simplified consensus sequence was determined by comparison of the sequence surrounding the recombination sites of the Hin, Gin, Cin and Pin systems, aligned here for maximum homology (Hiestand-Nauer and Iida, 1983; Plasterk *et al.*, 1983; Silverman and Simon, 1983). The consensus sequence used most often in this study was shorter than the simplified consensus suggested by Glasgow *et al.*, 1989 to reduce emphasis in the area where *S. typhimurium hixR* varied. Bases conserved between the recombination sites are underlined. The upper case letters represent bases within the inverted repeats of each system, the lower case letters indicate the bases outside of the inverted repeats in the ON orientation. Below the well-characterised recombination sites, the sequence of one of the proposed recombination sites for pilin gene inversion in *M. bovis* is shown (aligned for maximum homology with *hixL* (Marrs *et al.*, 1988, Marrs *et al.*, 1985). Redrawn from Glasgow *et al.* (1989).

Simplified consensus sequence:

-13 -1 +1 +13
TT-TC--AAACCAAGGTTT--GA-AA

Consensus sequence used in this study:

AAGGTTTTTTGATAAAG
G C AG A T

hixL attggTTCTTGAAAAACCAAGGTTTTTTGATAAagcaa

hixR taaaaTTTTCCTTTTGGAAGGTTTTTTGATAAccaat

gixL ACCGTTTCCTGTAAACCGAGGTTTTTGGATAAtggtc

gixR ACCGTTTCCTGTAAACCGAGGTTTTTGGATAAtggtc

cixL CCGAGTTCTCTTAAACCAAGGTTTAGGATtgaaatg

cixR CCGAGTTCTCTTAAACCAAGGTATTGGATaacagca

pixL gctccTTCTCCCAAACCAACgtttatgaaaatgaag

pixR gagccTTCTCCCAAACCAACgtttatgaaaatgaag

M. bovis ATTGCAGCTAGGATACCAATAATGGCGATAACAATC

ClaI-digested chromosomal *Bordetella* DNA. Several probes were investigated as there were several areas of variation in the DNA bases. Three probes were originally examined by MacGregor (1990); and one which he reported to hybridise was also used in this study (probe 1, Table 4.1.). For probe 2, where there was the possibility of a base variation, a G or C base was chosen in preference to an A or T as *Bordetella* DNA has a high GC ratio. Inclusion of information from *Moraxella bovis* suggested an alternative sequence (probe 3). Probe 4 was designed from *cix* information (Fig. 4.5., Section 1.9.1.c.) still emphasising the G,C content and including the *M. bovis* information. By examining the consensus sequence noted in Fig. 4.5, two longer probes were designed, again still emphasising the GC content (probes 5 and 6).

Hybridisation conditions are given in Section 2.7.17.c: Probes 1-4 were hybridised and membranes washed at 37°C, whereas probes 5 and 6 were hybridised and membranes washed at 45°C. Plasmid pKK1001B DNA and *S. typhimurium* DNA was digested with *BscI* and *StuI* while the other DNA was digested with *ClaI*. Probe 1 was found to hybridise to all the fragments of pKK1001B, several fragments in *S. typhimurium* (2 fragments >4 kb, 2.9, 0.92 and ~0.6 kb), two >4kb fragments in *E. coli* and one fragment >10 kb in the *B. pertussis* DNA. With the same probe MacGregor (1990) reported hybridisation to two large molecular weight fragments in *B. pertussis* but noted differences between strains: 14.5 kb and 12.5 kb *ClaI* fragments in Taberman DNA, and 18.6 and 16.5 kb *ClaI* fragments in Tohama DNA. Probe 2 (Fig. 4.6.) hybridised to ~620 bp, ~1 kb and ~4 kb fragments in pKK1001B (lane 1), multiple fragments in *S. typhimurium* DNA (lane 2) and a >10 kb and 2.2 kb fragment in the *B. pertussis* DNA (lanes 5-8; 10-12). DNA from *B. bronchiseptica* (lane 9) and *B. paraptussis* (lane 13) strains showed faint hybridisation to the same fragments. Hybridisation to *B. avium* DNA, p16 DNA and pIC20H containing 0.58 kb insert could not be seen (lane 14). Probe 3 showed more general hybridisation but two fragments of 1.1 kb and 1.95 kb in particular were noticeable in the DNA of *B.*

Table 4.1. Mixed sequence oligonucleotides constructed from related inverted repeats.

Probe 1.

A G G T T T A G G A A A A A G

C T T T T

Probe 2.

G A G G T T T A G G A A A A A G

C T T T

Probe 3.

G A G G T T G G G G A A A C G

C A C T

Probe 4.

G A G G T T G G G G A G A G

C A A C

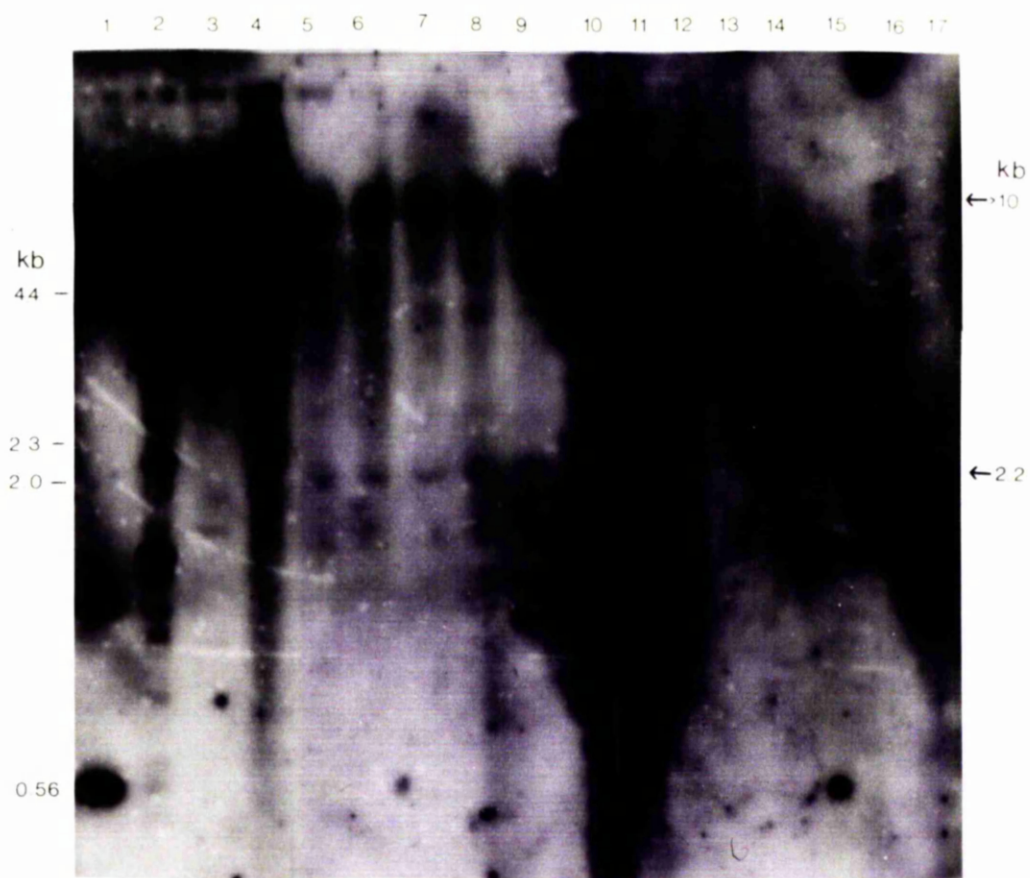
Probe 5. AAACCGAGGTTGGGGAGAG
 TTTGG C AA C

Probe 6. T G C C C G G A A C C G A G G T T G G G G A G A G
A C C T T T G G C A A C

The sequences are aligned to show areas of similarity. Where a base is shown below another, the oligonucleotide synthesised consisted of either base at that position.

Fig. 4.6. Southern blot of *Cla*I-digested *Bordetella* chromosomal DNA hybridised with oligonucleotide 2 (see Table 4.1) constructed from the conserved target sequence of the Hin-related inversion systems.

The lambda *Hind*III marker fragments are indicated on the left of the blot. The arrows to the right of the blot indicate fragments of interest. Lanes contained *Cla*I-digested DNA (except where indicated) prepared from the following plasmids and strains: (1) pKK1001B and (2) *S. typhimurium* LT2 (digested with *B*scI and *S*tuI). (3) *E. coli*; (4) *P. magnus* W1384; *B. pertussis*: (5) Taberman C-mode; (6) Tohama; (7) Wellcome 28; (8) L84 I; (10) Arg 40; (11) Arg 41; (12) L84 IV; (9) *B. bronchiseptica* 452; (13) *B. parapertussis* 5952; (14) *B. avium* 4148; (15) Clone p16 from *E. coli* (*dam*⁺), (16) Clone p16 from *E. coli* (*dam*⁻). (17) pIC20H containing 0.58 kb insert (digested with *Hind*III).



pertussis strains. Probe 4 (Fig. 4.7.) hybridised to multiple fragments in *S. typhimurium* DNA and very faintly to *E. coli* DNA. The probe also bound to two main fragments of >10 kb and >5 kb in the DNA from *B. pertussis* strains in addition to seven fragments in the range 3 kb-1.6 kb and one fragment of 1.1 kb size (lanes 3-7, 9-11). DNA from *B. bronchiseptica* (lane 8) and *B. parapertussis* (lane 12) strains showed similar hybridisation though non-specific hybridisation obscured some of the fragments. No hybridisation was found to the *B. avium* DNA, p16 and pIC20H containing 0.58 kb inserts apart from some non-specific hybridisation to the vector pLAFR1. The remaining two probes showed general non-specific hybridisation.

4.9. Discussion.

The hybridisation of the 680 bp probe to a ~0.58 kb fragment in *B. pertussis* DNA in this study confirmed the results of MacGregor (1990). However the degree of hybridisation was weak and the majority of the hybridisation was to a ~0.96 kb fragment. The absence of hybridisation to this larger fragment in MacGregor's work could have been due to differences in the probe or Southern transfer.

Hybridisation of the 680 bp probe to *Bordetella* DNA indicates homology to the *Hin* DNA contained in the probe. However this conclusion is not substantiated since the 620 bp probe also hybridised to the same *Bordetella* fragments, unless the 620 bp also consisted of similar sequences of DNA. A region of 47 bp at the N-terminus of the *hin* gene is not included in the 680 bp fragment and thus it is possible that the 620 bp fragment is upstream of the 680 bp fragment and includes these bases of the *hin* gene. If this were the case, both hybridisation results would suggest homology between the *Hin* DNA and *Bordetella* DNA.

Other explanations are possible for the hybridisation of both 680 bp and 620 bp DNA fragments to the *Bordetella* DNA. If the 620 bp was downstream of

Fig. 4.7. Southern blot of *Cla*I-digested *Bordetella* chromosomal DNA hybridised with oligonucleotide 4 (Table 4.1.) constructed from the conserved target sequence of the Hin-related inversion systems.

The lambda *Hind*III marker fragments are indicated to the left of the blot. The arrows to the right of the blots indicate fragments of interest. Lanes contained *Cla*I-digested DNA (except where indicated) prepared from the following plasmids and strains: (1) *S. typhimurium* LT2 (digested with *Bsc*I and *Stu*I). (2) *E. coli*;

B. pertussis: (3) Taberman C-mode; (4) Taberman X-mode; (5) Tohama; (6) Wellcome 28; (7) L84 I; (9) Arg 40; (10) Arg 41; (11) L84 IV; (8) *B. bronchiseptica* 452; (12) *B. parapertussis* 5952; *B. avium*: (13) 4148; (14) 4480; (15) Clone p16 from *E. coli* (*dam*⁺); (16) Clone p16 from *E. coli* (*dam*⁻). (17) pIC20H containing 0.58 kb insert (digested with *Hind*III).



the *hin* gene then no *Hin* DNA homologous sequences would be present in the 620 bp fragment. It is, however, possible that the 680 bp fragment actually consists of two fragments of very similar size which would explain its greater intensity as compared to the 620 bp fragment on ethidium bromide stained gels. One fragment would consist of the already recognised *Hin* DNA region (Fig. 3.2). The other could be an adjacent sequence. The *hin* gene is followed downstream by the *H2* flagellin promoter, the inverted repeat right, and then the *H2* flagellin gene (Fig. 3.2.). The DNA sequence of *H2* flagellin is known for the first 114 bp (Silverman *et al.*, 1981, Fig. 3.2.) and shows high homology to the fully sequenced *H1* flagellin gene (Joys, 1985, Appendix A.1.1). It is therefore possible that the *BscI* site present in the *H1* flagellin DNA sequence is also present in the *H2* flagellin sequence. If it occurs at the same site then another *StuI-BscI* fragment would be produced of 689 bp which, on electrophoresis would run close to the 678 bp *Hin* DNA containing band, resulting in the ~680 bp band containing more DNA than the front-running 620 bp band. The ~680 bp band would thus consist of 526 bp of the *hin* gene and also the *H2* gene promoter, right inverted repeat and part of the *H2* structural gene, thus confusing any hybridisation results.

The calculation of the distance from the *StuI* site to the proposed *BscI* site in the *H1* flagellin structural DNA, if it exists at same position as the *BscI* in the *H2* flagellin structural gene, is shown by addition of the following sizes. The distance from the *StuI* site (at the 3' end of the ~680 bp fragment which encloses the *Hin* DNA) to the inverted repeat right including the *H2* gene promoter = 180 bp; the inverted repeat right = 14 bp; the distance to the *H2* structural gene from the inverted repeat right = 16 bp and the possible distance in the *H2* flagellin DNA to the *BscI* site assuming the same location as in *H1* DNA = 479 bp. The total distance, $180 + 14 + 16 + 479 = 689$ bp.

The occurrence of the ~620 bp fragment can then also be explained by the presence of a *BscI* site in the *H1* flagellin DNA and the fact that on inversion

of the DNA the *BscI* site from the *Hin* DNA would then be closest to the H2 flagellin DNA resulting in a *BscI/BscI* fragment of 617 bp. Thus the ~620 bp fragment would contain 47 bp of the *hin* gene, the right inverted repeat and 479 bp of the H2 flagellum DNA. Thus, *BscI* site to the inverted repeat right = 108 bp; inverted repeat right = 14 bp; distance to H2 flagellin DNA' = 16 bp and the possible distance in H2 flagellin DNA to *BscI* site assuming same as H1 flagellin DNA = 479 bp. Total distance *BscI* to *BscI* = 108 + 14 + 16 + 479 = 617 bp.

Even if the site does not exist exactly as predicted, with perhaps a *StuI* site existing instead, the calculations show that it is possible for there to be a site which on digestion would produce fragments of ~680 bp and ~620 bp containing the same DNA sequence, enabling the same hybridisation pattern to occur with both fragments, as has been shown. The calculations would also explain why the 680 bp fragment appeared to consist of more DNA as it could consist of not only the *Hin* DNA region per genome but also the *H2* gene promoter, inverted repeat right and 479 bp of the *H2* flagellin gene. As the 620 bp would not consist of the *H2* gene promoter, any similarity of hybridisation must be due to the DNA invertase, inverted repeat and or flagellin region (N-terminus).

If the *BscI/StuI* site did not occur in the flagellin sequence, but upstream of the *hin* gene, this would still result in 620 and 680 bp fragments and they would not possess any homology with flagellin sequences. The alternative suggestion of the *BscI* site in the flagellin gene would enable both 680 bp and 620 bp probes to bind to the same fragment and the use of a flagellin probe should identify similar homologous fragments. These hypotheses were examined by the production of DNA fragments consisting only of the *hin* gene (Chapter 5) and flagellin gene sequences (Chapter 6) which were used as probes to the fixed chromosomal DNA.

Mixed oligonucleotides based on the invertase target sequence hybridised to several fragments in *ClaI*-digested *Bordetella* DNA. The GC codon usage may

have been over-emphasised, increasing the non-specific binding. Although the codon usage did reduce the variability of the bases, there were still various combinations of oligonucleotide sequences which could hybridise for each probe. Additional problems with mixed oligonucleotides of different compositions are that each of the hybrids melts at a different temperature and a high temperature may inhibit hybridisation in some cases whereas a low temperature may give non-specific hybridisation. Fewer fragments hybridised to probes which emphasised the *S. typhimurium* and *E. coli* target sequences rather than the *M. bovis* sequences, possibly showing more specificity to the former two organisms. The oligonucleotides did not hybridise to the same fragments as the 680 and 620 bp probes in *B. pertussis* DNA but rather to larger *Cla*I fragments.

CHAPTER 5

CHARACTERISATION OF A *B. PERTUSSIS* DNA FRAGMENT WHICH HYBRIDISED TO A *HIN* GENE PROBE.

5.1. Introduction.

The composition of the original Hin invertase DNA probe, 680 bp, (obtained from the digest of pKK1001B) was not fully known. The production of other invertase-specific probes was therefore investigated. The sequence of the *hin* DNA was known and it was therefore possible to amplify the gene directly from the plasmid pKK1001B or *S. typhimurium* chromosomal DNA using PCR. The amplified *hin* DNA, could then be used as a probe against the pKK1001B to check homology with the 620 and 680 bp fragments, but the main use of the *hin* DNA would be as a probe against *Bordetella* chromosomal DNA. This would also show if there was change in the hybridisation pattern compared to using the 680 bp and 620 bp fragment probes.

5.2. Common regions of homology in DNA invertase sequences for the construction of DNA probes.

The amino-acid sequence homologies between highly conserved regions of Hin-related recombinases are shown in Fig 5.1. The regions of homology chosen were from amino acid sequences which had the smallest variation in DNA bases when using the *Bordetella* codon usage (Locht and Keith, 1986; Maskell *et al.*, 1988). The amino acid sequences were used to design two DNA oligonucleotides on opposite DNA strands to be used as probes in hybridisations to *Bordetella* DNA. If the hybridisation proved successful, the oligonucleotides could then be used in a PCR reaction to amplify the DNA fragment from *Bordetella* chromosomal DNA.

Amino-acid sequence:

5' I G Y A R V 3'

DNA sequence: Oligonucleotide 1, 17 bp.

5'- ATC GGC TAC/T GCC/G CGC GT- 3'

Fig. 5.1. Comparison of the Hin-related recombinases, Min of plasmid p15B, Bin of *S. aureus* and TnpR of Tn3.

Part of the amino acid sequences of Hin (Zieg and Simon, 1980), Gin (Plasterk *et al.*, 1983), Cin (Hiestand-Nauer and Iida, 1983), Pin (Plasterk *et al.*, 1983), Min (Iida *et al.*, 1990), Bin (Rowland and Dyke, 1988) and TnpR (Heffron and McCarthy, 1979) are aligned with a gap for maximum homology; redrawn from Glasgow *et al.* (1989) with additional information on Min. The common amino-acid sequences (1 and 2) used to construct the two DNA oligonucleotides are shown in bold, the second oligonucleotide was from the inverted complementary strand. The numbers at the top of the figure represent the amino-acid numbers of Hin from the beginning of the open reading frame.

a.a.	1		64
Hin	MATI-GYIRV	--->	LDRLGR
Gin	VL I-GYVRV	--->	LDRLGR
Cin	MLI-GYVRV	--->	LDRLGR
Pin	MLI-GYIRV	--->	LDRLGR
Min	MQI-GYIRV	--->	LDRLGR
Bin	LKI-GYARV	--->	LDRLGR
TnpR	MRIFGYARV	--->	LDRLGR
	1) IGYARV	---> 2)	LDRLGR

Amino-acid sequence:

5' L D R L G R 3'

DNA sequence of inverted complimentary strand: Oligonucleotide 2, 18 bp.

5' GCG GCC CAG GCG GTC CAG 3'

Labelling of the oligonucleotides and hybridisation conditions are given in Sections 2.7.16.b and 2.7.17.c. respectively. The temperature used for hybridisation with both probes was 45°C. Oligonucleotide 1 was found to hybridise only faintly to all the *Cla*I-digested *B. pertussis* DNA preparations whereas oligonucleotide 2 showed stronger hybridisation to the chromosomal digests with a common banding pattern in *B. pertussis* and *B. bronchiseptica* although varying in *B. avium* (Fig. 5.2). The *B. parapertussis* DNA did not digest to completion so hybridisation patterns could not be compared. In the controls, hybridisation was found to pKK1001B and generally to the *S. typhimurium* and *E. coli* DNA with more predominant bands showing amongst general streaming.

The absence of hybridisation by the oligonucleotide 1 and the non-specific binding of oligonucleotide 2 suggested that these probes would not be useful in locating a homologous DNA invertase fragment. A further probe was therefore investigated.

5.3. *Hin* gene amplified from *S. typhimurium* DNA by PCR.

To produce a more specific probe, the *S. typhimurium* *Hin* invertase DNA was alone examined. The nucleotide sequence was analysed for the flanking regions of the *hin* gene (as shown in Fig. 3.2). The sequence was used to design two oligonucleotides in 5' to 3' orientations from complementary strands for PCR amplification of the *hin* gene from plasmid pKK1001B and *S. typhimurium* chromosomal DNA. These oligonucleotides would be more specific than those used in Section 5.2. as only the *S. typhimurium* sequence was used in their

Fig. 5.2. Southern blot of *Bordetella* chromosomal DNA hybridised with oligonucleotide 2 designed from regions of homology common to invertases.

The mol. wt. (kilobase) markers (Gibco-BRL) are indicated to the left of the blot. Lanes contained DNA from: (1) pKK1001B and (2) *S. typhimurium* digested with *BscI* and *StuI*; (3) *E. coli*, (4) *Peptostreptococcus magnus* W1384; *B. pertussis* strains: (5) Taberman, (6) Tohama, (7) W28, (8) L84I, (9) L84 IV, (10) Arg 40, (11) Arg 41, (12) *B. bronchiseptica* 452, (13) *B. parapertussis* 5952, (14) *B. avium* 4148 and (15) *B. avium* 4480 digested with *ClaI*.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

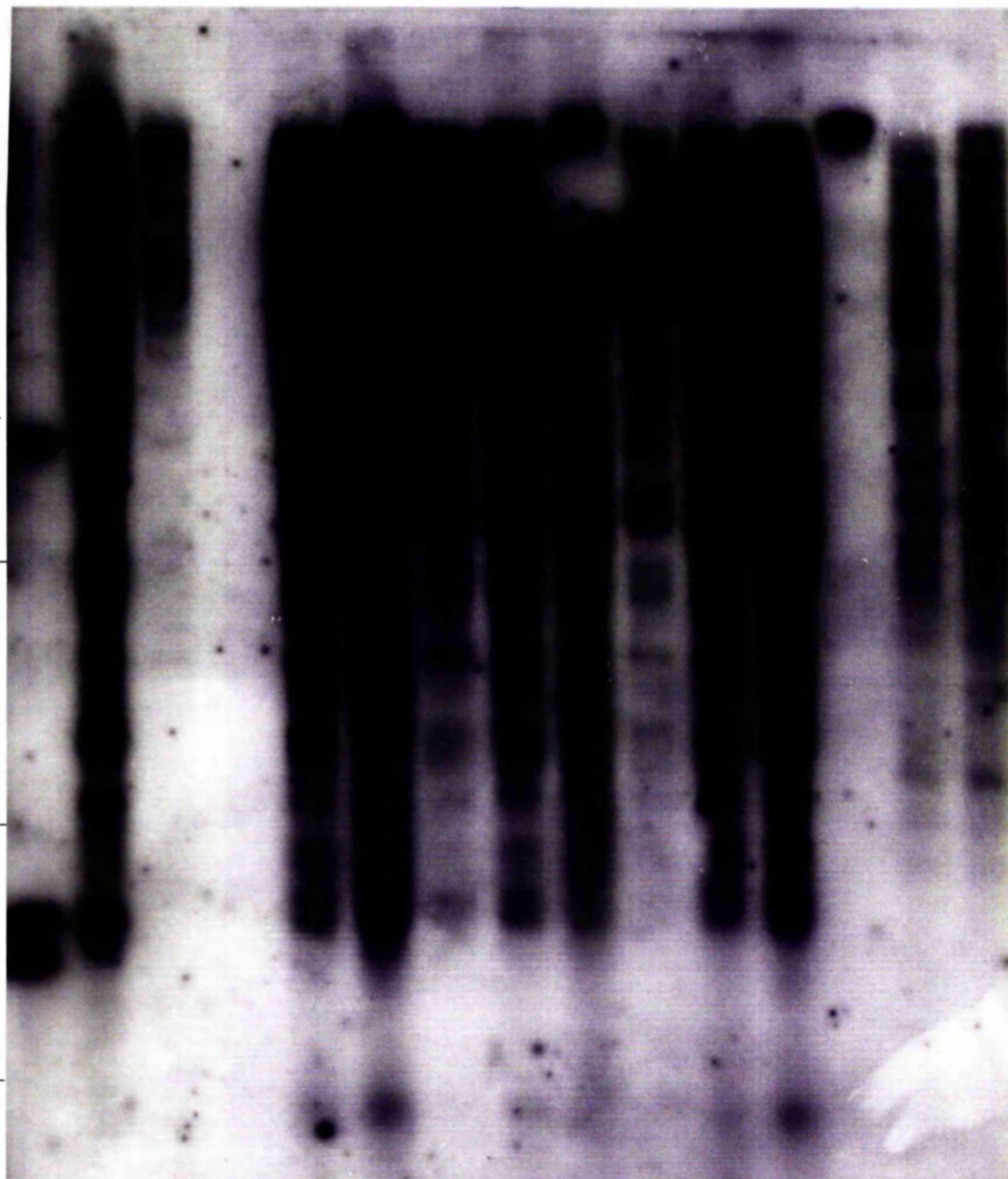
kb

3.0—

2.0—

1.0—

0.5—



construction. The oligonucleotides were:

Oligo (1):

5'- TCT GAT GTA AAG GAG AAA ATC - 3'

Oligo (2):

5'- GAT TTT TAT TTT AAT TCA TTC - 3'

The PCR reaction (2.7.14.) enabled the amplification of the *hin* gene from both the pKK1001B and chromosomal *S. typhimurium* DNA. The PCR product, a 604 bp fragment in both cases, shown after electrophoresis on a 1% agarose gel (Fig. 5.3.). pKK1001B was chosen as the main source of DNA for PCR as it resulted in a higher concentration of amplified DNA.

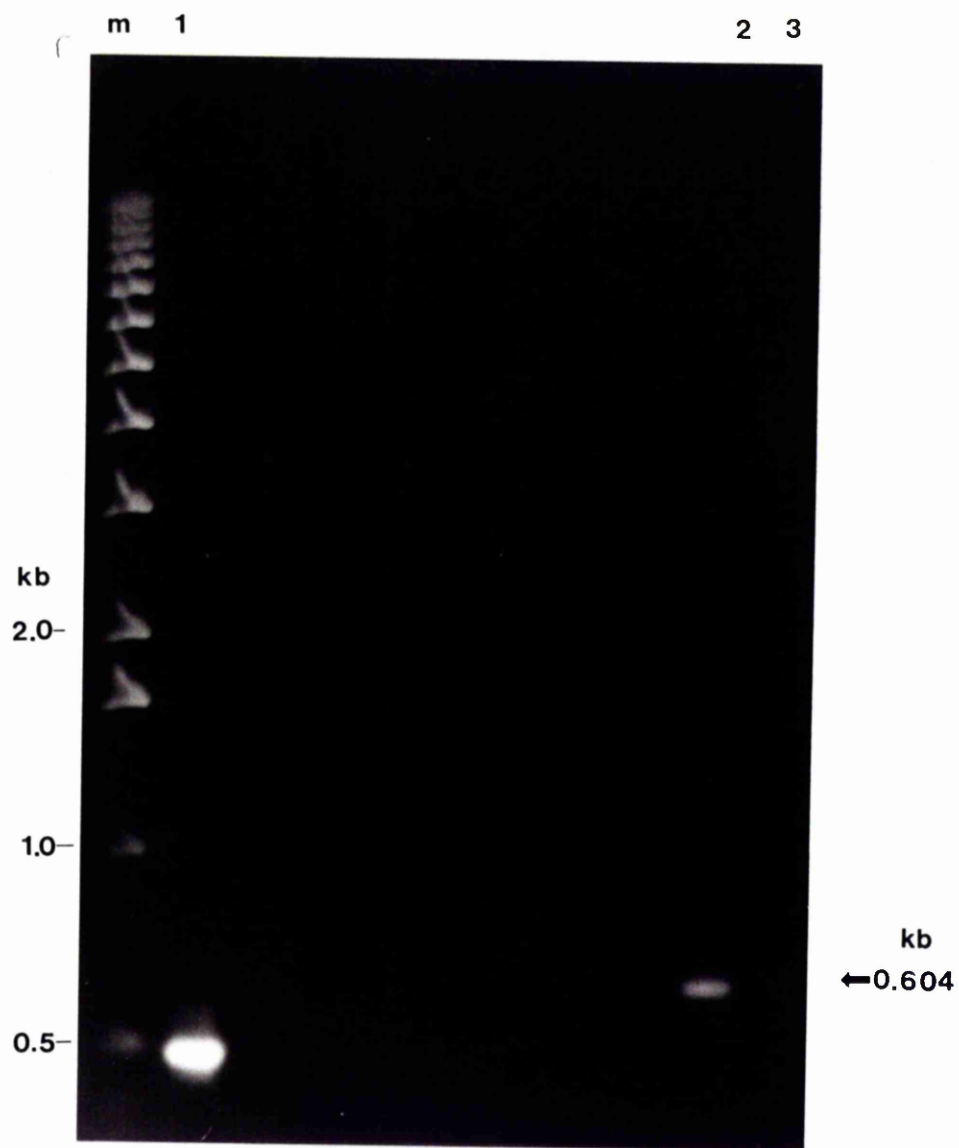
The oligonucleotides were also used in PCR reactions under the same conditions with *Bordetella* chromosomal DNA. However no specific fragment was produced, only multiple fragments > 10 kb.

5.4. Digestion of pKK1001B with *BscI*/*StuI*/*SacI* and hybridisation with *hin* gene probe.

To ensure that digestion of pKK1001B DNA, described in Chapter 4, had gone to completion sequential digestions were performed in various combinations to see if the same fragments were produced. Hybridisation with the amplified *hin* gene probe would then show if there was homology to the *hin* gene in both the 620 bp and the 680 bp DNA fragment from pKK1001B. The *hin* probe was hybridised to Southern blotted pKK1001B DNA digested with various combinations of enzymes. The enzymes tested included *BscI*/*ClaI* and *StuI*, followed by digestion with *SacI*. There is a known *SacI* site within the invertase (Fig 3.2.) and also within the remaining *S. typhimurium* DNA in pKK1001B (Fig. 3.1).

Fig. 5.3. Electrophoresis of the PCR amplified *hin* DNA fragment from pKK1001B and *S. typhimurium* chromosomal DNA.

The kilobase (Gibco-BRL) marker is shown (m) on the left of the photograph. Lane (1) shows control DNA amplified (0.5 kb) from the GeneAmp PCR reagent kit (Perkin Elmer Cetus); (2) *hin* DNA amplified from pKK1001B and (3) *hin* DNA amplified from *S. typhimurium* chromosomal DNA.



Single digests were performed initially with *BscI*, *ClaI*, *StuI* and *SacI*. Double digestions included digestions by *BscI* then *StuI*, *ClaI* then *StuI*, *StuI* then *BscI*, *StuI* then *ClaI*. Where sequential digestions required enzymes to be in different buffers, the DNA was precipitated with ethanol after the first digestion then resuspended in sterile distilled water and the second buffer added. The triple digestions covered the various combinations: *BscI*, *StuI* and *SacI*; *ClaI*, *StuI* and *SacI*; *StuI*, *BscI* and *SacI* and *StuI*, *ClaI* and *SacI*. No differences were seen between the products from any of these restriction digests (Fig. 5.4).

The gel (Fig. 5.4.) was Southern blotted and hybridised with radiolabelled *hin* gene (Section 2.7.16.a.). There were no differences between the hybridisation patterns of fragments produced by digestions in the various orders (Fig. 5.5.). Interestingly the *BscI/ClaI* and *StuI* digests showed hybridisation of the *hin* probe mainly to the 680 bp fragment but also to the larger fragment indicating that this digestion did not result in all the *hin* DNA being present within the 680 bp (lanes 6,7,8,9). There was also some hybridisation to the 620 bp DNA because it is also contained *S. typhimurium* DNA and may have also consisted of some *hin* DNA (see Chapter 4, Discussion). Inclusion of *SacI*-digestion produced hybridisation to two smaller fragments presumably originating from digestion of the 680 bp fragment, as would be expected (lanes 10,11,12,13) if the 680 bp fragment contained *hin* DNA which has a *SacI* site. Not all of the ~680 bp fragment was digested, further suggesting that the fragment does not solely consist of the *hin* DNA.

5.5. Hybridisation of *ClaI*-digested chromosomal *Bordetella* DNA with the *hin* gene probe.

The *hin* DNA was used as a probe against Southern-blotted *ClaI*-digested *Bordetella* chromosomal DNA (Fig. 5.6). The probe hybridised to all fragments in pKK1001B (lane 1). It also hybridised in the 620 bp to 680 bp region, to 1.85

Fig. 5.4. Digestion of pKK1001B with various combinations of *BscI*, *StuI* and *SacI*.

The sizes of the lambda *HindIII* markers (m) are indicated on the left of the gel. The arrows on the right of the gel indicate fragments of particular interest. Lanes contained pKK1001B DNA digested with the following enzymes: (1 and 2) *BscI*; (3) *ClaI*; (4) *StuI*; (5) *SacI*; (6) *BscI* and *StuI* (7) *ClaI* and *StuI*; (8) *StuI* and *BscI*; (9) *StuI* and *ClaI*; (10) *BscI*, *StuI*, *SacI*; (11) *ClaI*, *StuI*, *SacI* (12) *StuI*, *BscI*, *SacI* (13) *StuI*, *ClaI*, *SacI*.

Fig. 5.5. Southern blot of pKK1001B digested with various combinations of *BscI*, *StuI* and *SacI* (Fig. 5.4.) and hybridised with the PCR-amplified *hin* DNA probe.

The lambda *HindIII* marker is indicated to the left of the blot. The arrows to the right of the blot indicate fragments of particular interest. Lanes contained plasmid DNA pKK1001B digested as shown in Fig. 5.4.

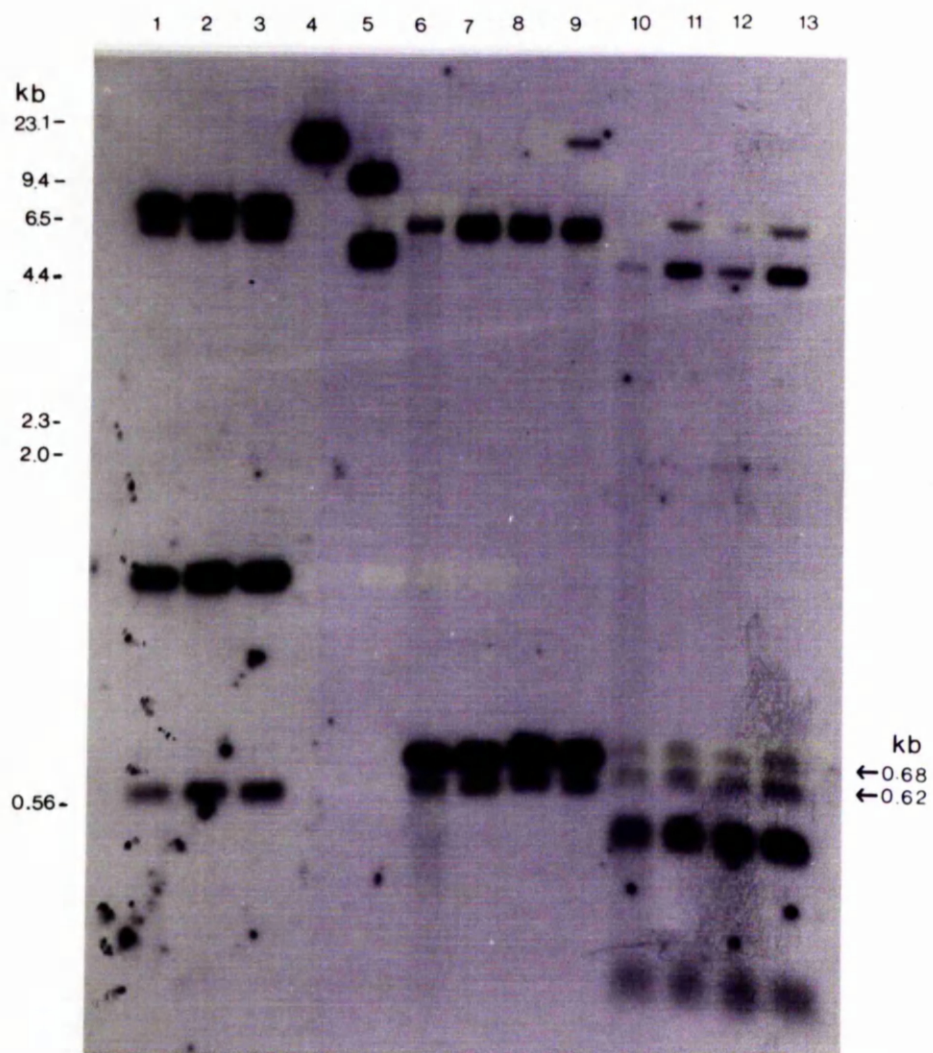
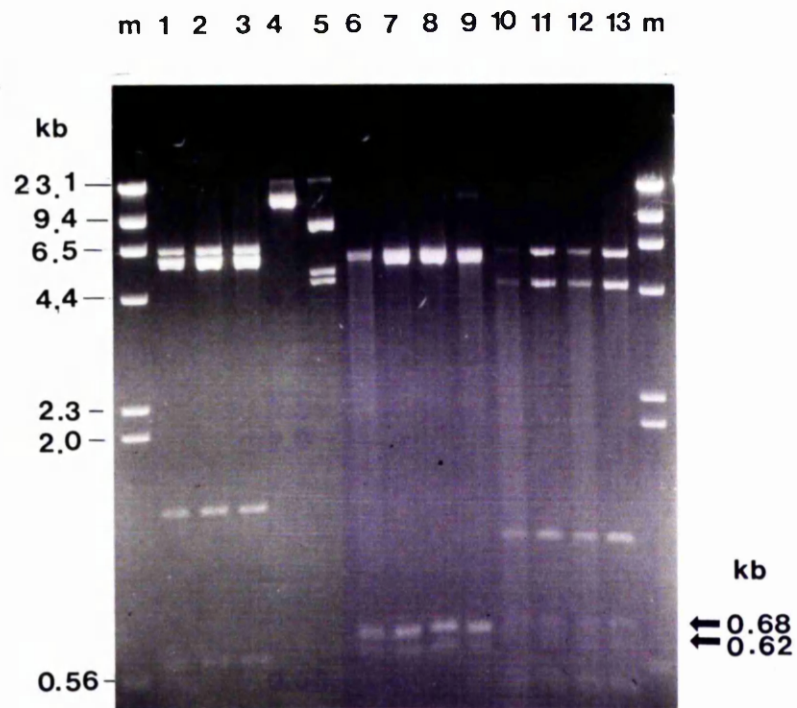
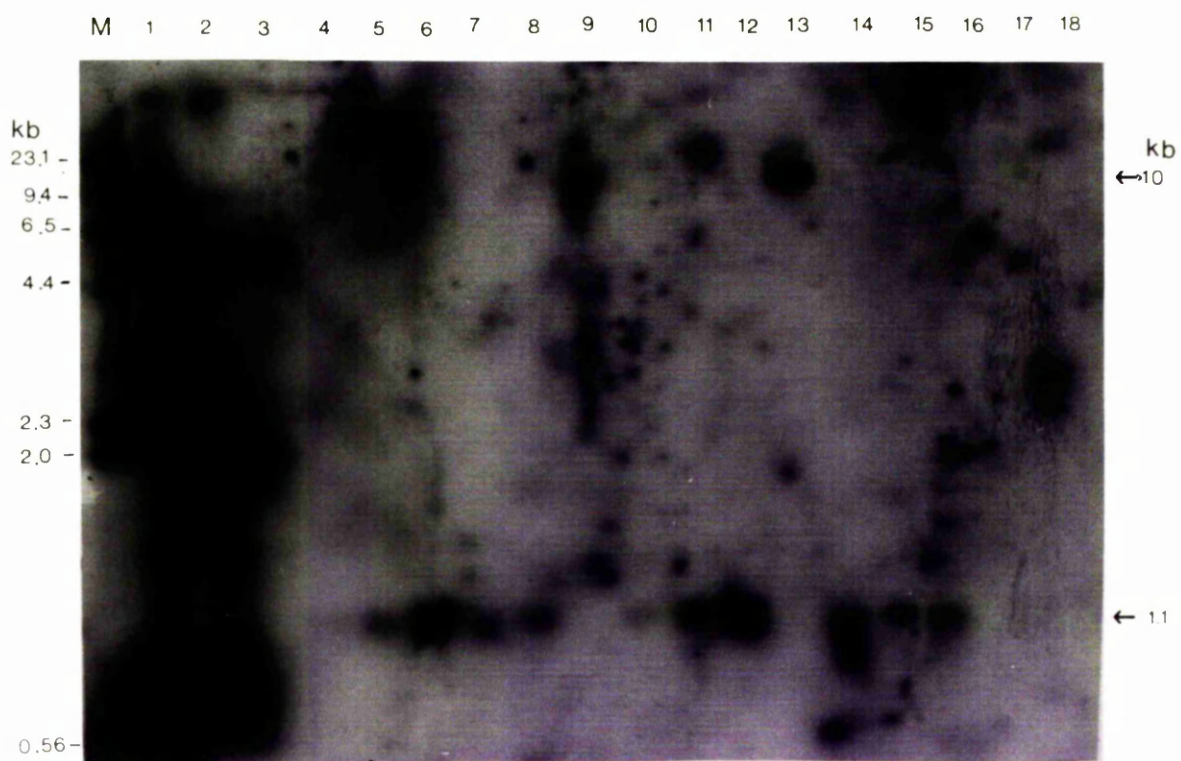


Fig. 5.6. Southern blot of *Cla*I-digested *Bordetella* chromosomal DNA hybridised with PCR-amplified *hin* DNA probe.

The lambda *Hind*III markers (m) are indicated to the left of the blot. The arrows to the right of the blot indicate fragments of interest. Lanes contained DNA prepared from the following plasmids or strains digested with *Cla*I (except where indicated): (1) pKK1001B and (2) *S. typhimurium* LT2 (digested with *Bsc*I and *Stu*I). (3) *E. coli*;

B. pertussis: (4) Taberman "C mode"; (5) Taberman; (6) Tohama; (7) Wellcome 28; (8) L84 I; (10) Arg 40; (11) Arg 41; (12) L84 IV; (9) *B. bronchiseptica* 452; (13) *B. parapertussis* 5952; *B. avium*: (14) 4148; (15) 4480; (16) Clone p16 from *E. coli* (*dam*⁺). (17) Clone p16 from *E. coli* (*dam*⁻).

(18) pIC20H containing 0.58 kb insert (digested with *Hind*III).



kb and ~5 kb fragments of *S. typhimurium* (lane 2), and to a ~5 kb fragment of *E. coli* (lane 3). The hybridisation to the *B. pertussis* (lanes 4-8, 10-12) and *B. avium* DNA (14,15) was to fragments of ~1.1 kb whereas for *B. parapertussis* and *B. bronchiseptica*, the hybridisation was to a fragment > 10 kb (lanes 9 and 13). There was no hybridisation to the 0.58 kb *ClaI* fragment (lanes 16, 17 and 18) originally noted as homologous to *Hin* invertase DNA (MacGregor, 1990).

5.6. Chromosomal DNA of *B. pertussis* Taberman (X-mode and C-mode) digested with *ClaI* or *SalI* and hybridised with i) pKK1001B 680 bp fragment and ii) the *hin* PCR-amplified gene probe.

To determine if the 680 bp and the *hin* DNA probes were hybridising to the same fragments, they were used in sequential hybridisations on the same blot. *Bordetella* chromosomal DNA was digested with *ClaI* or *SalI*, Southern blotted and then hybridised with the 680 bp probe. After exposure, the blot was stripped of hybridised DNA (Section 2.7.19.) and rehybridised with the *hin* DNA obtained by the PCR reaction. Hybridisation with the 680 bp probe showed homology to the ~ 0.96 kb fragment on *ClaI* digestion (Fig. 5.7.a., lanes 1 and 4) and to a ~ 1.6 kb fragment on *SalI* digestion (lanes 2 and 5). However, hybridisation to larger fragments were also noted in both digestions. No differences were found between the DNA of strain Taberman grown under X-mode or C-mode conditions. A further blot (data not shown) of *ClaI* and *SalI*-digested DNA of strains L84 I and L84 IV gave the same hybridisation patterns including the larger sized fragments as Taberman DNA when probed with the 680 bp probe. These larger fragments were not noted on previous blots and could be due to the probe or incomplete complete digestion of the *Bordetella* DNA.

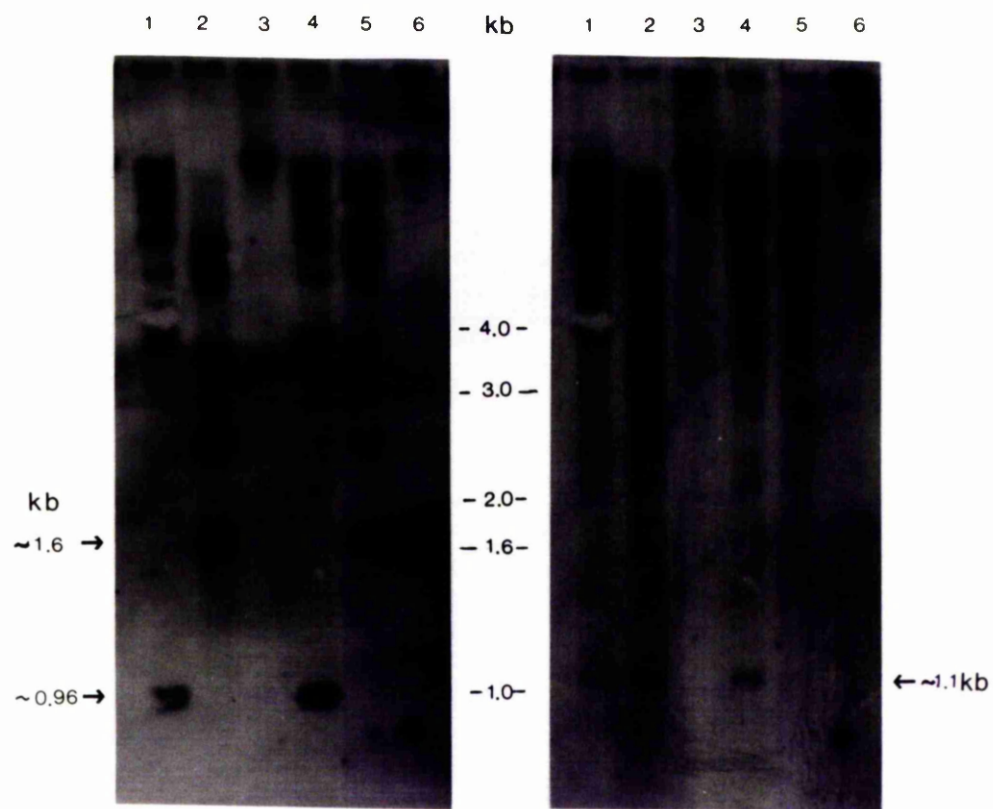
On stripping the membrane and rehybridising with the *hin* PCR-amplified gene probe, hybridisation occurred to a ~1.1 kb *ClaI* fragment (Figure 5.7.b. lanes 1 and 4) and a ~1.1 kb fragment on *SalI* digestion (lanes 2 and 5). *BamHI*

Fig. 5.7. Southern blot of *Cla*I and *Sal*I-digested *Bordetella* DNA hybridised with (a) 680 bp fragment (from pKK1001B digested with *Bsc*I and *Stu*I); and (b) PCR-amplified *hin* DNA.

The mol. wt. (kilobase) markers (Gibco-BRL) are indicated between the blots. The arrows to the left and right of the blots indicate fragments of interest. Lanes contained Taberman DNA (C or X mode) digested with the following enzymes:

Taberman (C-mode) digested with (1) *Cla*I; (2) *Sal*I; (3) *Bam*HI

Taberman (X-mode) digested with (4) *Cla*I; (5) *Sal*I; (6) *Bam*HI



a

b

was found not to digest the DNA and so gave no more information. The two probes were therefore hybridising to different fragments, the *Cla*I fragments were very similar in size, but digestion with *Sal*I enabled a distinction to be drawn between them.

The *hin* probe was apparently hybridising to a ~1.1 kb fragment of *Cla*I-digested *B. pertussis* DNA. Ethidium bromide staining of agarose gels containing *Cla*I-digested *B. pertussis* DNA shows the appearance of a repeat region which stands out as an intensely staining band (not shown). Analysis of the sequence of a tandem repeat in the *B. pertussis* genome (Glare *et al.*, 1990, Appendix A.1.2.) has shown the presence of a *Cla*I and a *Sal*I site so that digestion with *Cla*I or *Sal*I results in multiple copies of 1.046 kb fragments. The *hin* DNA may thus have homology to this repeat sequence. Hybridisation may also have been due to the fact that the tandem repeat sequence was at a higher concentration and a GC-rich area might enable preferential hybridisation to occur. The sequences of the *hin* probe and the *B. pertussis* tandem repeat were therefore examined for any homology.

5.7. Sequence comparison of *hin* gene and *B. pertussis* tandem repeat. The region of homology, 17 bp, used to design a probe for hybridisation to *Bordetella* chromosomal DNA.

The tandem repeat sequence and the PCR amplified *hin* DNA sequence were compared (using DNASTAR) to determine the extent of homology. The tandem repeat sequence of Glare *et al.* (1990) was adapted from the homologous insertion sequence reported by McLafferty *et al.* (1988) which was available on the computer database and differed only by a few bases. The *hin* DNA sequence and the tandem repeat sequence were compared using the programme Align, and the largest regions of homology were also apparent using the Dotplot programme. This most homologous sequence occurred as 15 bp within a 17 bp sequence (Fig. 5.8, N^o1). The Dotplot also showed another equivalent area of

Fig. 5.8. DNA sequence comparison of *B. pertussis* tandem repeat and PCR amplified *hin* DNA.

The tandem repeat sequence (Glare *et al.*, 1990) and the *hin* DNA sequence (Silverman *et al.*, 1981) were compared using the programme Align. The most homologous sequences were N^o1 and N^o2. Both regions represent 75% identity over 20 bases. The underlined bases represent homologous bases between the two sequences.

Nº 1.

Tandem repeat: 440 C CTGGCCGA C CTGGAGCGGC 459

PCR amplified *hin*: 354 A CTGGCCGA G A TGGAGCGAG 373

Nº 2.

Tandem repeat: 356 GGCTGAC CCCA AGCGCGCATG 375

PCR amplified *hin*: 405 GGCTGCCG CCAGAGCGCAAG 424

homology (Fig. 5.8, N^o2). These regions represent 75% homology over 20 bases. The regions appeared to be GC-rich, although the *hin* DNA from *S. typhimurium* generally should not be as G-C rich as the *Bordetella* DNA to which it was hybridising. The sequence of the *S. typhimurium hin* DNA was altered to the GC codon usage of *Bordetella* and again compared to the repeat sequence by Dotplot analysis. The number of homologous regions was increased to fifteen with 75% identity and three with 80% identity over 20 bases. One of these latter sequences was the same as before increasing the G-C codon usage i.e. (Fig. 5.8. N^o1). This region is G-C rich and perhaps is an area showing the particular homology to bind the probe. It is possible that the hybridisation of the *hin* probe could be due to this small GC-rich region in both DNA sequences allowing hybridisation at low stringency. Although the *hin* DNA is ~600 bp, it was labelled by the Multiprime system which would allow the labelling of smaller fragments such as the 17 bp fragment. A 17 bp oligonucleotide was designed from the homologous tandem repeat sequence (Fig 5.8. N^o1):

5' CTG GCC GAC CTG GAG CG 3'

This was used as a probe to a Southern blot of *Cla*I-digested *Bordetella* DNA (Fig. 5.9). It hybridised strongly to the tandem repeat area of *B. pertussis* DNA (1.1 kb) and also presumably to repeats elsewhere in the chromosome to give a streaming effect. Hybridisation to *B. bronchiseptica* and *B. parapertussis* was found only in the higher mol. wt. region (lanes 9 and 13). No hybridisation was seen to the *B. avium* DNA (lanes 14 and 15) possibly due to insufficient DNA. The probe also hybridised in the area of the ~620 bp to ~680 bp region and generally within the *S. typhimurium* chromosomal DNA.

After stripping the blot and rehybridising with the *hin* DNA, hybridisation was found to occur to the same areas in the *B. pertussis* DNA but there was also strong hybridisation to the pKK1001B and *S. typhimurium* DNA. The use of an oligonucleotide exactly as found in the *hin* 17 bp and not that of the repeat as was used here could have been more useful. However, hybridisation with the

Fig. 5.9. Southern blot of *Cla*I-digested *Bordetella* chromosomal DNA hybridised with 17 bp probe derived from the *B. pertussis* tandem repeat sequence.

The lambda *Hind*III markers are indicated to the left of the blot. The arrows to the right of the blot indicate fragments of interest. Lanes contained DNA prepared from the following plasmids or strains digested with *Cla*I (except where indicated): (1) pKK1001B and (2) *S. typhimurium* LT2 (digested with *Bsc*I and *Stu*I). (3) *E. coli*; (4) *Peptostreptococcus magnus* W1384; *B. pertussis*: (5) Taberman; (6) Tohama; (7) Wellcome 28; (8) L84 I; (10) Arg 40; (11) Arg 41; (12) L84 IV; (9) *B. bronchiseptica* 452; (13) *B. parapertussis* 5952; (14) *B. avium* 4148; (15) 4480; (16) Clone p16 from *E. coli* (*dam*⁺); (17) Clone p16 from *E. coli* (*dam*⁻). (18) pIC20H containing 0.58 kb insert (digested with *Hind*III).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

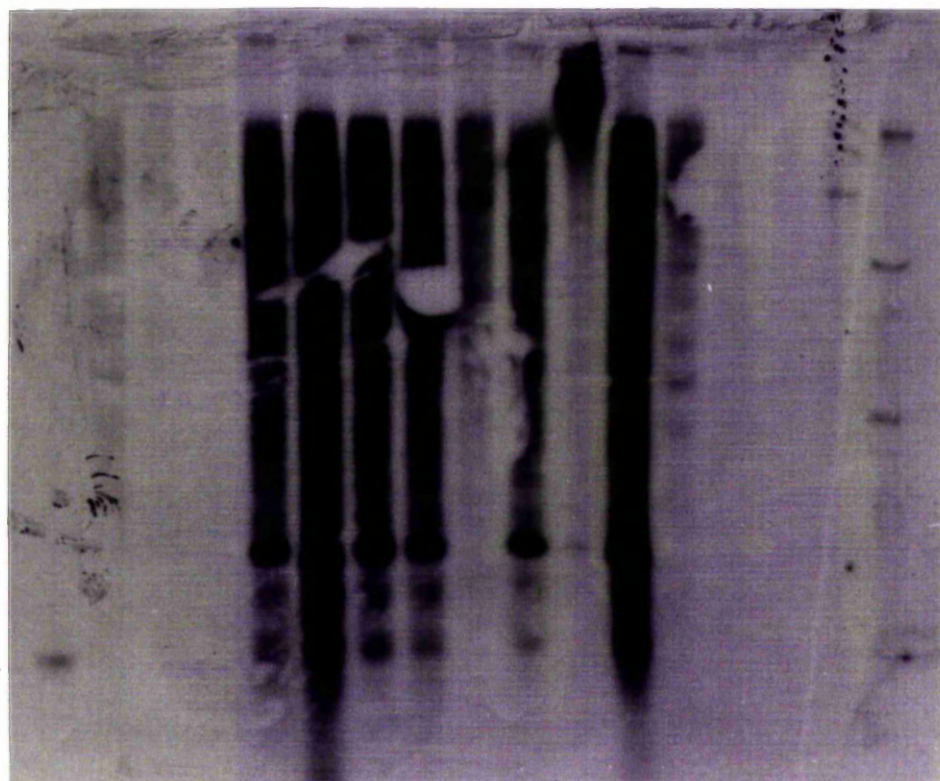
kb

23.1 —

4.4 —

2.0 —

0.56 —



kb

← 1.1

← 0.58

whole *hin* DNA probe to the same blot produced hybridisation in the same place, aiding interpretation as to the similarity in hybridisation between the *hin* and tandem repeat probes.

5.8. Discussion.

The hybridisation of the *hin* DNA to *B. pertussis* *Cla*I-digested chromosomal DNA (1.1. kb) was similar to the hybridisation using the 680 bp fragment (~ 0.96 kb) but was not as strong. The slight difference between the sizes of the fragments to which the two probes hybridised suggested that the two probes were binding to different fragments. This was confirmed by digestion of the *Bordetella* DNA with a different enzyme, *Sal*I. The 680 bp fragment probe appeared to consist of other DNA fragment(s) in addition to the invertase DNA which enabled strong hybridisation to a different *Bordetella* fragment.

The *hin* DNA hybridised to fragments in *B. pertussis* DNA recognised as tandem repeats. Sequence comparison of *hin* and the tandem repeat DNA showed small areas of strong homology. One of these areas, a 17 bp sequence, was chosen to show that this could have sufficient homology to allow hybridisation to occur. Interestingly, the *hin* DNA probe was shown to hybridise to the other *Bordetella* species. The *B. avium* strain showed homology with the same sized *Cla*I fragment as in *B. pertussis* (Section 5.5), but this repeat does not occur in *B. avium* strains. It is possible that multiple copies are not necessary for hybridisation. The hybridisation in *B. bronchiseptica* and *B. parapertussis* strains was to *Cla*I fragments of > 10 kb. The sequence of the *B. pertussis* 1.05 kb tandem repeat has been identified as a transposon-like insertion element, IS481 (McLafferty *et al.*, 1988). This tandem repeat has not been noted in the other *Bordetella* species. It is possible that the homology shown here in these strains is due to the presence of a single insertion sequence (IS). The location of ISs is also interesting as ISs have been shown to occur close to *B. pertussis* virulence genes. One is located upstream of the adenylate cyclase locus and another is

located immediately adjacent to the C-terminal end of the agglutinin 2 fimbrial subunit gene (McPheat *et al.*, 1989b) and one close to the porin gene (Li *et al.*, 1991b).

These results suggested that the *hin* DNA probe was not hybridising to a homologous DNA invertase sequence in *B. pertussis* but rather to an insertion sequence, tandemly repeated. The absence of tandem repeats in the other species suggests that it is not necessary for the insertion sequence to be in multiple copies to enable hybridisation. However, this would suggest the presence of insertion sequences within the other *Bordetella*. Single copy bands have been shown in DNA from *B. bronchiseptica* (McPheat and McNally, 1987a; Glare *et al.*, 1990) and *B. parapertussis* (McPheat and McNally 1987a) when hybridised with IS homologous probes. Insertion sequences have also been found in *B. parapertussis*, *B. bronchiseptica* by Zee *et al.* (1993) and *B. avium* by Savelkoul *et al.* (1993). If the homology in this study does represent the presence of insertion sequences in the other bordetellae it would be interesting to discover which genes they are close to on the chromosome.

The hybridisation of the 620 bp and 680 bp *S. typhimurium* fragments of pKK1001B to *Bordetella* DNA as had been suggested in Section 4.9. Discussion might also be due to flagellin gene homology. This was examined in Chapter 6.

CHAPTER 6

ANALYSIS OF *BORDETELLA* DNA FOR HOMOLOGY TO FLAGELLIN GENE SEQUENCES.

6.1. Introduction.

Within the four species of *Bordetella*, two are non-motile: the human pathogens *B. pertussis* and *B. parapertussis*; and two are motile: the broad host-range mammalian pathogen *B. bronchiseptica*, and the avian pathogen *B. avium*. The motility of the latter two species is due to peritrichous flagella (Pittman, 1984a; Kersters *et al.*, 1984). In Chapters 4 and 5, hybridisation was seen between *Bordetella* DNA and restriction digest fragments of the plasmid pKK1001B that could have contained *S. typhimurium* H2 flagellin DNA sequences. A DNA probe consisting of only flagellin gene sequences was used in a hybridisation to Southern-blotted chromosomal *Bordetella* DNA to see if there was homology to the *S. typhimurium* flagellin.

The complete *H1* flagellin gene has been sequenced (~ 1.47 kb; Joys, 1985, Appendix A.1.1.) but only 114 bp of the *H2* flagellin gene sequence has been published (Silverman *et al.*, 1981). A restriction enzyme map of the gene could yield a *H1* flagellin DNA probe. Fig. 6.1. shows the alignment of the first 114 bp of the two DNA sequences of *H1* and *H2* flagellin genes indicating their close similarity. Thus a probe consisting of the *H1* gene could conceivably detect *H2* DNA sequences and any homologous *Bordetella* DNA sequences by hybridisation. The *H1* gene could be prepared by amplifying the DNA from *S. typhimurium* chromosomal DNA using PCR. An oligonucleotide probe could also be designed from the conserved N-terminal amino acid sequences of various flagellin proteins; e.g. *S. typhimurium*, *E. coli* and *B. bronchiseptica* (Akerley *et al.*, 1992).

6.2. Examination of partially-sequenced *S. typhimurium* H2 flagellin DNA for restriction sites.

Examination of the 114 bp sequence of H2 flagellin DNA (Silverman *et al.*, 1981) shows the first restriction site to occur at the 5' end is *BbvI*. Analysis of restriction sites downstream of the *BbvI* site in H2 flagellin DNA (Zieg *et al.*,

Fig. 6.1. Alignment of H1 and H2 flagellin DNA sequences.

H1 flagellin DNA sequence, bases 13 to 1485 (1473 bp) (Joys, 1985)
aligned with H2 flagellin DNA sequence, bases 1036 to 1149 (114 bp)
(Silverman *et al.*, 1981) .

	20v	30v	40v	50v	60v	70v
(<u>H1</u>):	ATGGCACAAGTCATTAAATACAAACAGCCTGTCGCTGTTGACCCAGAATAACCTGAACAAA					
	ATGGCACAAGT AT AA AC AACAG CTGTCGCTG TGACCCAGAATAACCTGAACAAA					
(<u>H2</u>):	ATGGCACAAGTAATCAACTAACAGTCTGTCGCTGCTGACCCAGAATAACCTGAACAAA					
	1040^	1050^	1060^	1070^	1080^	1090^

	80v	90v	100v	110v	120v	130v
(<u>H1</u>):	TCCCAGTCCGCTCTGGGCACCGCTATCGAGCGTCTGTCTTCCGGTCTGCGTATCAACAGC					
	TCCCAGTCCGC CTGGGCACCGCTATCGAGCGTCTGTCTT C GGCTGCGT C					
(<u>H2</u>):	TCCCAGTCCGCACTGGGCACCGCTATCGAGCGTCTGTCTTCTGGTCTGCGTTCC					
	1100^	1110^	1120^	1130^	1140^	

1978) shows an *Eco*RI site ~1.1 kb away. A *Bbv*I /*Eco*RI digest should thus cover the majority of the *H2* flagellin gene. However, as the majority of the sequence between the sites was not known, other *Bbv*I sites may be present which would have affected the result. The plasmid pKK1001B was digested with *Bbv*I in a single digest, and in a double digest with *Eco*RI. Both digests gave multiple fragments showing that there were multiple *Bbv*I sites and the desired single fragment could not be obtained.

As part of the sequence was known, it would have been possible to amplify the 114 bp of the *H2* flagellin gene from the pKK1001B by PCR. However, the known sequence was only the N-terminal, a tenth of the whole gene. As an alternative, the *H1* flagellin gene was amplified from *S. typhimurium* DNA as the whole of its sequence was known.

6.3. Design of flagellin probes.

Two types of flagellin probe were prepared for hybridisation to *Bordetella* chromosomal DNA in Southern blots.

Probe 1. From the *H1* gene sequence (Joys, 1985, Appendix A.1.1.) two oligonucleotides were derived for use in the PCR reaction to amplify the entire *H1* flagellin structural gene (1.47 kb) from *S. typhimurium* chromosomal DNA.

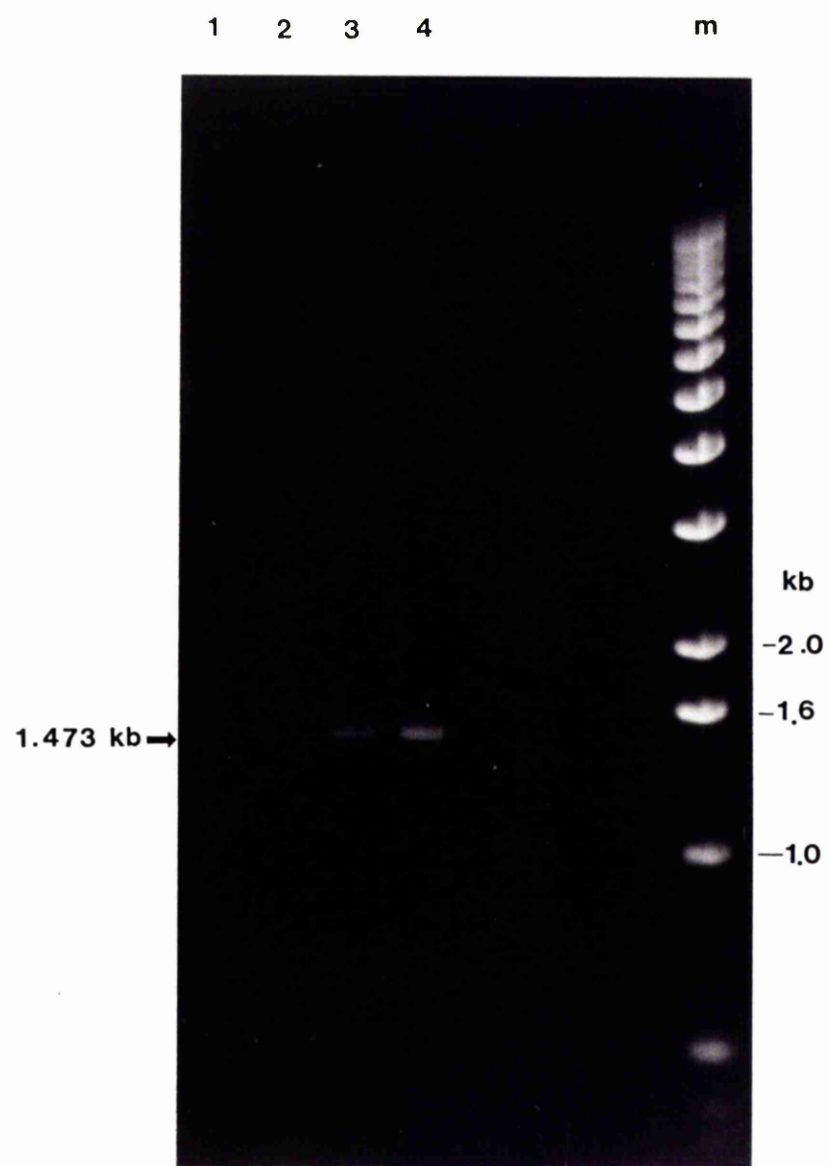
- A. 5' - ATG GCA CAA GTC ATT AAT ACA- 3' and
- B. 5' - TTA ACG CAG TAA AGA GAG GAC- 3'.

PCR reactions were as in Section 2.7.14. The PCR product was checked by electrophoresis on a low melting point 1% agarose gel (Fig. 6.2.), the *H1* flagellin DNA fragment cut from the gel and the DNA radiolabelled in the agarose (Section 2.7.16.a).

Probe 2. A 15 bp oligonucleotide was designed from the conserved N-terminal amino acid sequences of various flagellin proteins, those of *S. typhimurium*, *E. coli* and *B. bronchiseptica* (Akerley *et al.*, 1992). The *Bordetella*

Fig. 6.2. Electrophoresis of the PCR-amplified *H1* flagellin gene from *S. typhimurium* chromosomal DNA.

The sizes of the mol. wt. (kilobase, Gibco-BRL) marker (m) is shown to the right of the gel. The arrow to the left marks the amplified *H1* flagellin gene. Lanes contained (1) no amplification of *H1* flagellin gene from pKK1001B, (2,3,4) amplification of *H1* flagellin gene from *S. typhimurium* chromosomal DNA (2) 200 ng, (3) 400 ng and (4) 800 ng initially present in PCR reaction.



codon usage noted by Locht and Keith (1986) and Maskell *et al.* (1988) was used to derive the DNA sequence from the amino acid (aa) sequence.

aa sequence : Val Ile Asn Thr Asn

DNA sequence: 5' - GTC/G ATC AAC ACC AAC- 3'

The oligonucleotide was synthesised and radiolabelled as in Sections 2.7.15. and 2.7.16.b.

6.4. Hybridisation of *ClaI*-digested *Bordetella* DNA with *S. typhimurium* H1 flagellin gene probe.

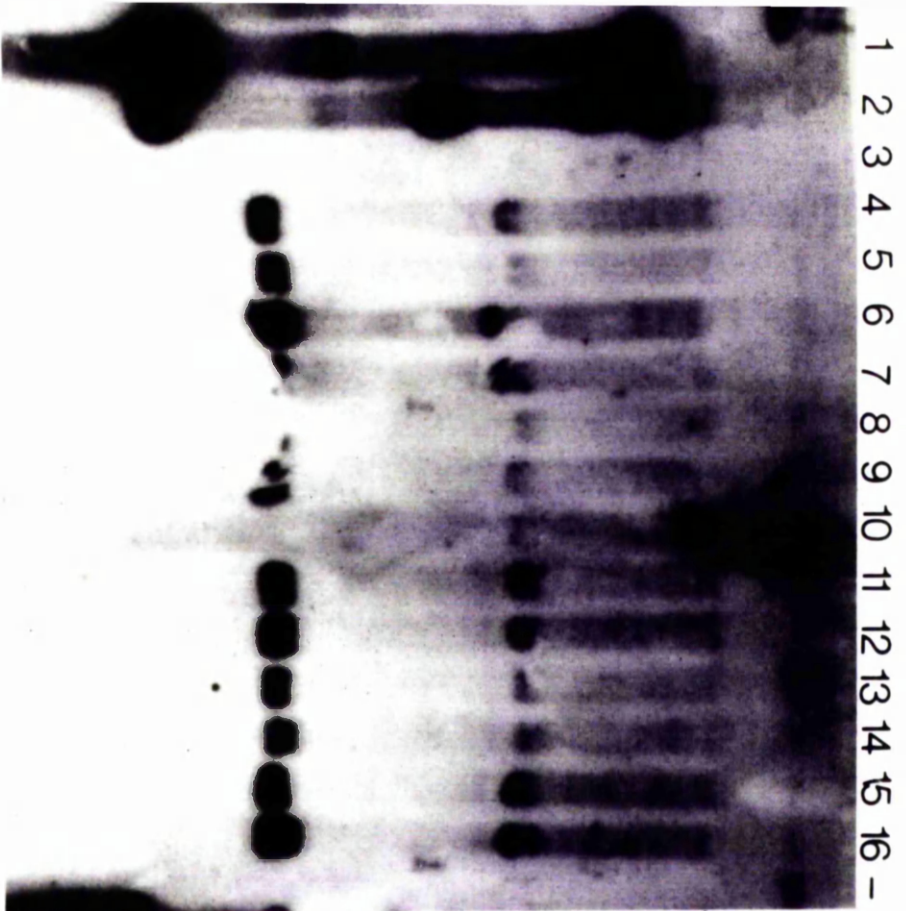
Membranes of Southern-blotted chromosomal DNA were initially hybridised with DNA probe 1 after which the probe was removed from the membrane (Section 2.8.19.) and the membrane was then re-hybridised with probe 2. Hybridisation conditions for probe 1 and probe 2 are given in Sections 2.7.17.a. and 2.7.17.c. respectively.

Southern blots of *ClaI*-digested chromosomal DNA from bordetellae showed some variation in the pattern of hybridisation between the four species when hybridised with the *S. typhimurium* H1 flagellin gene probe. The probe hybridised to a fragment (> 10 kb) which was common to all strains of *B. parapertussis* and *B. bronchiseptica* (Fig. 6.3. lanes 10, 23-31) but was absent in *B. pertussis* (lanes 4-9, 11-16, 20-22) and *B. avium* strains (lanes 32-33). *B. parapertussis* strain 10520 (lane 24) varied in also having a fragment > 13 kb. *B. parapertussis* and *B. bronchiseptica* also appeared to share a 2.8 kb fragment with all strains of *B. pertussis* tested. A ~0.96 kb fragment which hybridised in all strains of *B. pertussis* and *B. avium* tested, was absent in the *B. parapertussis* and *B. bronchiseptica* digests. The probe hybridised to all fragments in the digest of pKK1001B (lanes 1 and 17) and to four fragments (0.65, 1.85, 3.6, and ~6.1kb:

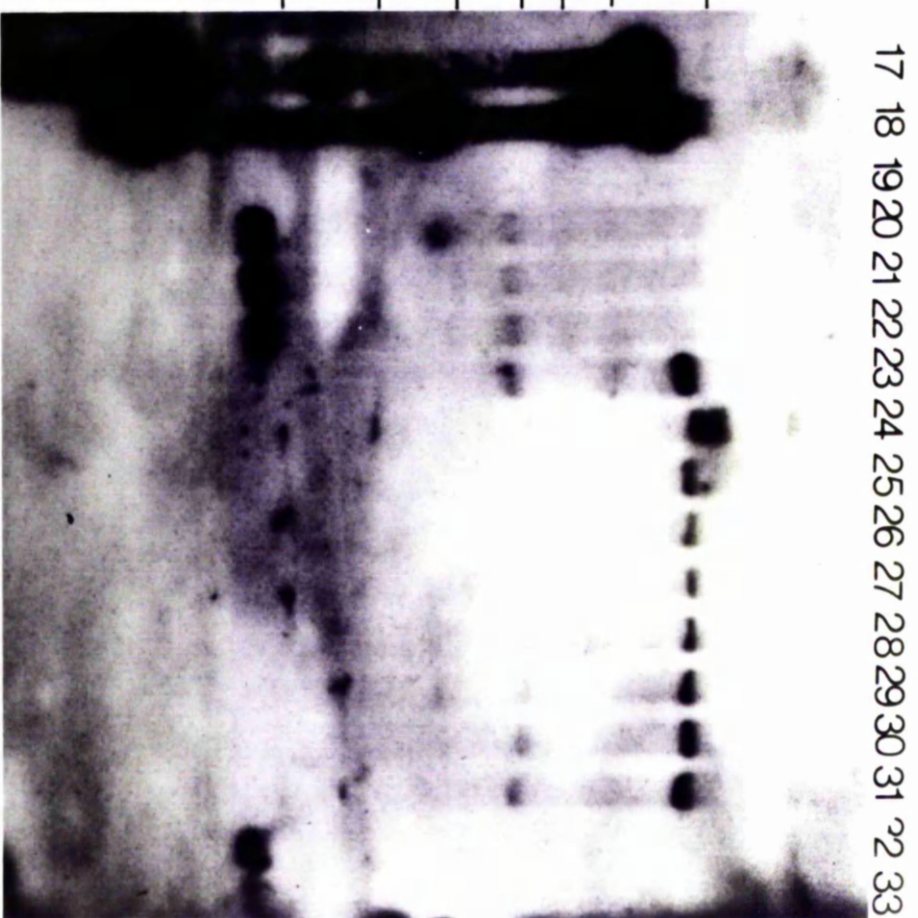
Fig. 6.3. Southern blot of *Cla*I-digested *Bordetella* chromosomal DNA hybridised with the PCR-amplified *H1* flagellin gene of *S. typhimurium*.

The mol. wt. (kilobase) markers (Gibco-BRL) are indicated between the blots. The arrows to the right of the blots indicate fragments of particular interest. Lanes contained DNA prepared from the following plasmids or strains digested with *Cla*I (except where stated): (1 and 17) pKK1001B and (2 and 18) *S. typhimurium* LT2 (digested with *Bsc*I and *Stu*I). (3 and 19) *Peptostreptococcus magnus* W1384;

B. pertussis: (4) Taberman; (5) Taberman "C mode"; (6) Tohama; (7) Wellcome 28; (8) Arg 40; (9) Arg 41; (11) L84 I; (12) L84 IV; (13) 134 I; (14) 134 IV; (15) D 30042 I; (16) D 30042 IV; (20) 11615 IV; (21) M2 I; (22) M2 I; *B. parapertussis* (23) 5952; (24) 10520; *B. bronchiseptica*: (10 and 25) 452; (26) A51 I; (27) A51 III; (28) 276 I; (29) 276 III; (30) FW5 I ; (31) FW5 III. *B. avium* : (32) 4148; (33) 4480.



-12.0-
 -5.0-
 -4.0-
 -3.0-
 -2.0-
 -1.6-
 -1.0-



← 10
 ← 2.8
 ← 0.5

lanes 2 and 18) of DNA from *S. typhimurium* (Gram-negative, flagellate) but there was no hybridisation to DNA from *P. magnus* (Gram-positive, non-flagellate: lanes 3 and 19).

6.5. Hybridisation of *Cla*I-digested DNA with flagellin oligonucleotide probe.

The membranes used in Fig. 6.3. were stripped of the *H1* flagellin gene probe and reprobbed with the 15 bp oligonucleotide (probe 2; Fig. 6.4). This hybridised to several fragments >4kb in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. The 2.8 kb fragment which appeared common to these strains with the *H1* flagellin gene probe was also apparent with the oligonucleotide probe. The *B. pertussis* strains could be distinguished from the *B. parapertussis* and the *B. bronchiseptica* strains by the presence of a 3.7 kb DNA fragment in *B. pertussis* strains, and a 3.2 kb fragment in the *B. parapertussis* and *B. bronchiseptica* strains. Homology to a ~0.96 kb fragment was again apparent in *B. avium*, but was not readily visible in other strains and was somewhat obscured by stronger hybridisation to a 1.1 kb fragment in most strains except *B. avium*. In the tracks containing the control DNA, the level of hybridisation to pKK1001B was greatly reduced, *S. typhimurium* showed four DNA fragments (0.68, 1.75, 1.95, ~5.2kb) that hybridised to the probe, but again there was no hybridisation to DNA from the non-flagellate *P. magnus*.

6.6. Hybridisation of *Sal*I-digested *Bordetella* DNA with *S. typhimurium* *H1* flagellin gene probe.

Southern blots of *Sal*I-digested chromosomal DNA from bordetellae, when hybridised with the *S. typhimurium* *H1* flagellin gene, showed increased distinction in the pattern of variation between the four species (Fig. 6.5), although homology between the majority of strains of each species was noted, as with the *Cla*I preparation.

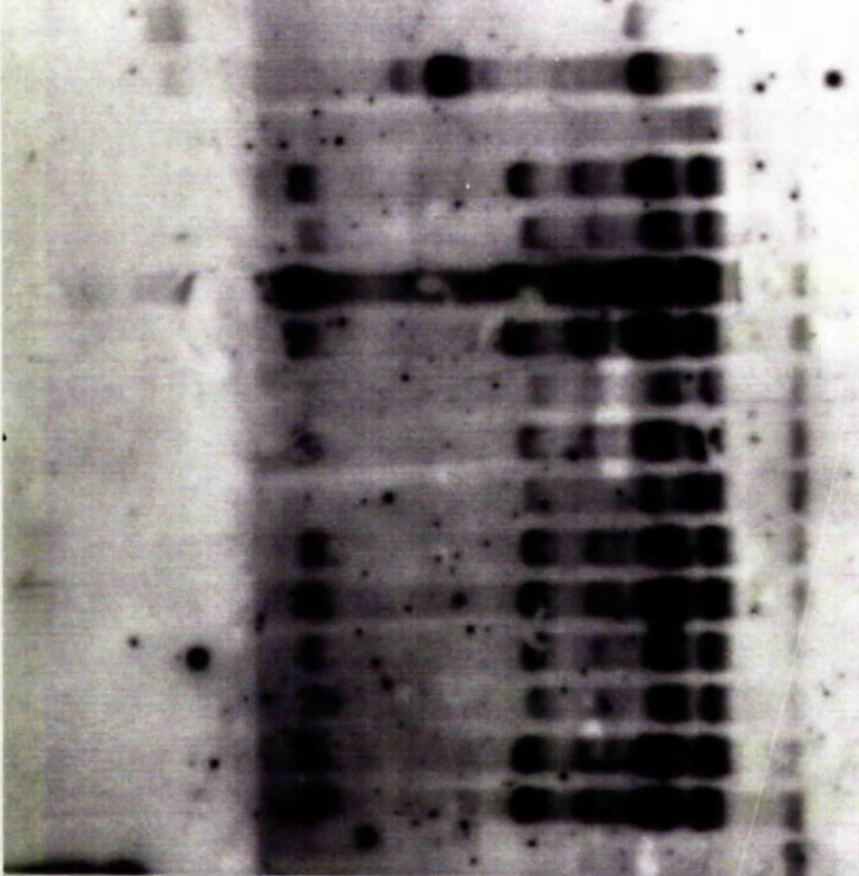
The probe hybridised to one fragment (~5.6 kb) in *B. bronchiseptica*

Fig. 6.4. Southern blot from Fig. 6.3. stripped of the probe and rehybridised with the radiolabelled 15 bp oligonucleotide derived from the conserved N-terminal flagellin sequences.

The mol. wt. (kilobase) markers (Gibco-BRL) are indicated between the blots. The arrows to the right of the blots indicate fragments of particular interest. Lanes contained DNA prepared from the following plasmids or strains digested with *Cla*I (except where stated): (1 and 17) pKK1001B and (2 and 18) *S. typhimurium* LT2 digested with (*Bsc*I and *Stu*I). (3 and 19) *Peptostreptococcus magnus* W1384;

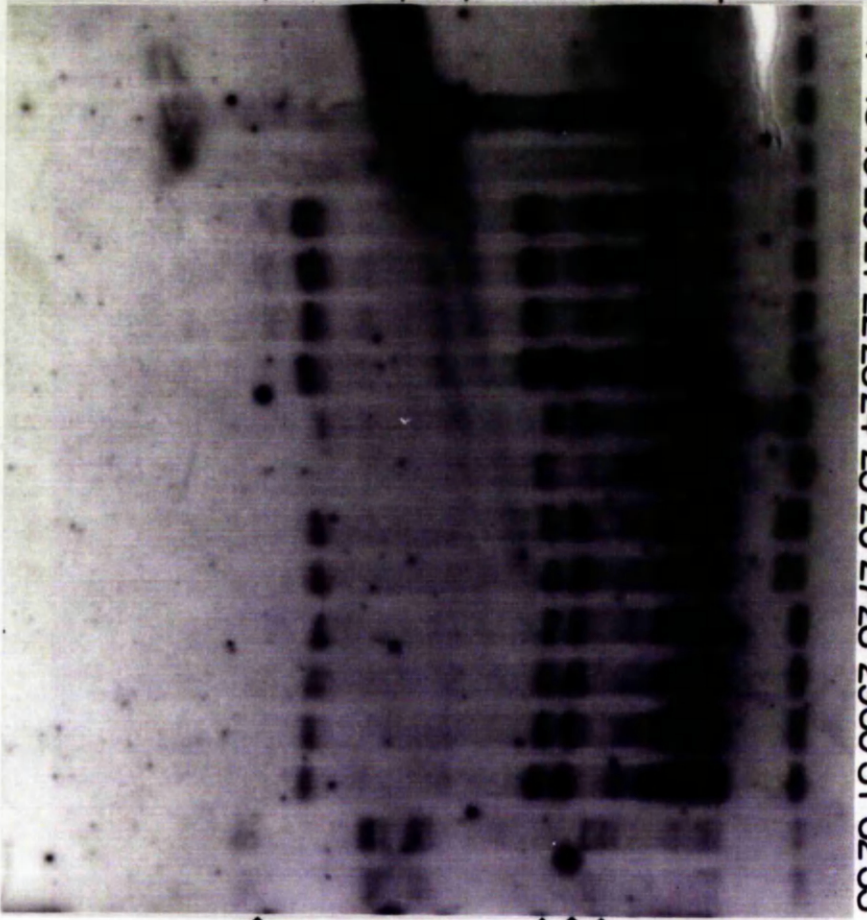
B. pertussis: (4) Taberman; (5) Taberman "C mode"; (6) Tohama; (7) Wellcome 28; (8) Arg 40; (9) Arg 41; (11) L84 I; (12) L84 IV; (13) 134 I; (14) 134 IV; (15) D 30042 I; (16) D 30042 IV; (20) 11615 IV; (21) M2 I; (22) M2 I; *B. parapertussis* (23) 5952; (24) 10520; *B. bronchiseptica*: (10 and 25) 452; (26) A51 I; (27) A51 III; (28) 276 I; (29) 276 III; (30) FW5 I ; (31) FW5 III. *B. avium* : (32) 4148; (33) 4480.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 -



kb
-12.0-
-5.0-
-4.0-
-3.0-
-2.0-
-1.6-
-1.0-

17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

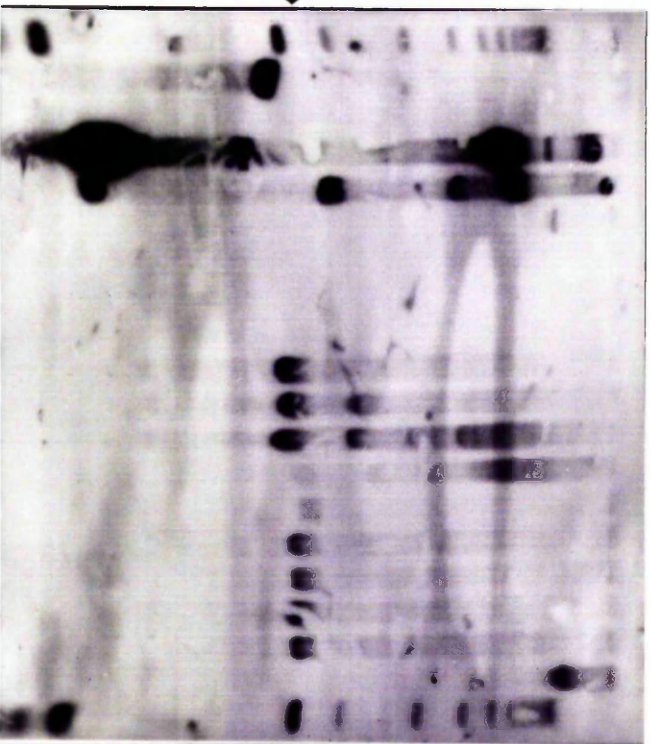


kb
←3.7
←3.2
←2.8
←0.96

Fig. 6.5. Southern blot of *SalI*-digested *Bordetella* chromosomal DNA hybridised with the PCR-amplified *H1* flagellin gene of *S. typhimurium*.

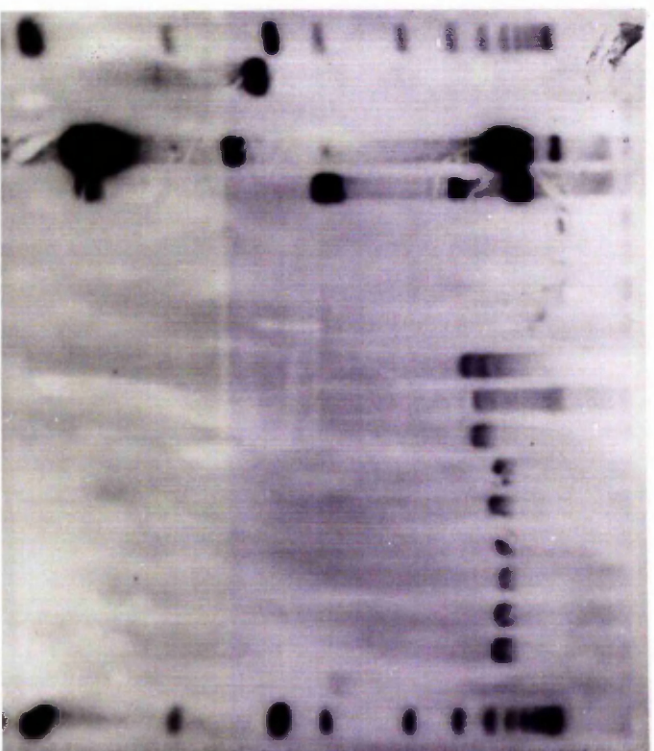
The mol. wt. (kilobase) markers (Gibco-BRL) are indicated between the blots. The arrows to the left and right of the blots indicate fragments of particular interest. Lanes contained DNA prepared from the following plasmids or strains all digested with *SalI* (except where stated): (1 and 18) *H1* flagellin gene amplified by PCR; (2 and 19) pKK1001B and (3 and 20) *S. typhimurium* LT2 (digested with *BscI* and *StuI*). (4 and 21) *E. coli*; (5 and 23) *Bacillus stearothermophilus*; (6 and 24) *Clostridium acetobutylicum*; (7 and 22) *Peptostreptococcus magnus* W1384; *B. pertussis*: (8) Taberman; (9) Taberman "C-mode"; (10) Tohama; (11) Wellcome 28; (12) Arg 40; (13) Arg 41; (14) L84 I; (15) L84 IV; (16) D 30042 I; (17) D 30042 IV; *B. parapertussis*: (25) 5952; (26) 10520; *B. bronchiseptica*: (27) 452; (28) A51 I; (29) A51 III; (30) 276 I; (31) 276 III; (32) FW5 I; (33) FW5 III; (34) *B. avium* 4148.

m 1 - 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 m



kb
~1.65 →

m 18 - 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 m



kb
-6.0-
-5.0-
-4.0-
-3.0-
-2.0-
-1.6-

kb
←~5.6
←~4.9
←~2.2

strains (lanes 28-33) except for strain *B. bronchiseptica* 452 (lane 27) where the one fragment was ~4.7 kb though this may be due to variation in the depth of the gel affecting the electrophoresis of the samples in this area. The two *B. parapertussis* strains 5952 and 10520 (lanes 25 and 26) also showed similar sized fragments of ~4.9 kb. *B. parapertussis* 10520 was again seen to differ from the strain 5952 by having two fragments, the additional one of 4.3 kb (lane 26). Differences between the two strains had also been noted previously on *Cla*I digestion of the *B. parapertussis* DNA (Fig. 6.3. lanes 23 and 24). There was no common fragment in the *B. pertussis* strains. A fragment common to *B. bronchiseptica*, *B. parapertussis* and *B. pertussis* was noted after *Cla*I digestion.

The *H1* flagellin gene probe hybridised to a ~1.65 kb *Sal*I fragment in all strains of *B. pertussis* (Fig. 6.5. lanes 8-16), except (lane 17) where D30042 IV did not digest with *Cla*I. Taberman DNA prepared from the strain originally grown under C-mode conditions appears to have an extra fragment of 2.1 kb (lane 9) and Tohama DNA has extra fragments of 2.1, 2.8 and 3.7 kb but this variation may have been caused by the DNA not being completely digested (lane 10). The *B. avium* fragment is ~2.2 kb (lane 34) varying with the *B. pertussis* fragment size in contrast to the fragment size similarity which had been noted in the *Cla*I digestion of these species (Fig. 6.3).

The probe again hybridised to all *Bsc*I/*Stu*I fragments in the digest of pKK1001B and to four fragments in *S. typhimurium* DNA as noted with the other blots. This blot shows the hybridisation to a *Sal*I fragment > 12 kb in *E. coli* DNA (Gram-negative, flagellate) but no hybridisation was detected to DNA from *Bacillus stearothermophilus* (Gram-positive, flagellate) or to DNA from *Clostridium acetobutylicum* (Gram-positive, flagellate) or to DNA from *Klebsiella aerogenes* (data not shown) (Gram-negative, non flagellate).

6.7. Discussion.

The pKK1001B 680 bp fragment hybridised to the same sized *Cla*I and *Sal*I fragments of *B. pertussis* DNA as did the *H1* flagellin gene probe. Therefore, the hybridisation noted with the 680 bp probe in Chapter 4 could possibly have been due to flagellin gene sequences. Although the original impetus for the flagellin work was to corroborate this idea, the work has provided other useful information on homology to flagellin gene sequences in all species of *Bordetella*.

The flagellin DNA probes, both the whole *S. typhimurium* *H1* flagellin gene and the 15 bp oligonucleotide derived from the conserved N-terminal sequences of various flagellins, showed homology to DNA from all four species of *Bordetella* despite only two of them being motile. This indicates the presence of non-expressed flagellin genes in the two non-motile species (*B. pertussis* and *B. parapertussis*). Other workers, in a recent report (Akerley *et al.*, 1992) mentioned that *B. pertussis* chromosomal DNA contained sequences which hybridised to degenerate oligonucleotide probes based on the *B. bronchiseptica* flagellin amino acid sequences. This work supports that observation and extends the investigation to non-motile *B. parapertussis* strains and motile *B. avium* strains which also possess homology with the *S. typhimurium* flagellin gene sequences.

In the *Cla*I digests, those fragments shown to have homology to the *H1* DNA probe also appeared to hybridise to the oligonucleotide probe although the hybridisation of the oligonucleotide to the 0.96 kb fragment was not apparent except with *B. avium*. The probe consisting of the whole *S. typhimurium* *H1* flagellin gene hybridised to fewer *Bordetella* chromosomal DNA fragments than did the oligonucleotide probe designed from the conserved flagellin N-terminus. With the *S. typhimurium* chromosomal DNA, three hybridising fragments were of similar size with both probes although a band in each was different. These results can be explained in part by the smaller size of the

N-terminal oligonucleotide and the emphasis on the high GC codon usage of *Bordetella*. This would lead to the increased possibility of non-specific hybridisation to *Bordetella* sequences not related to the flagellin sequence, whereas the lower GC of the *S. typhimurium* DNA would result in the *H1* probe binding more strongly than the oligonucleotide to the flagellin structural genes. It has been shown that two classes of flagellins are produced by *B. bronchiseptica* strains although only one type is expressed by any one strain (Akerley *et al.*, 1992). It is possible that the extra bands in the *Bordetella* strains which hybridise to the probe might signify the presence of more than one flagellin structural gene. There were no differences detected in the hybridisation patterns of *B. pertussis* strains, irrespective of phase, antigenic mode, serotype or origin. The *B. bronchiseptica* blots were also the same irrespective of the phase or origin of the strains. *B. parapertussis* showed slight variation between the two strains, but this might have been due to methylation of one strain or incomplete digestion of chromosomal DNA. For *B. avium*, again no variation was detected between the strains.

The presence of non-expressed genes has been shown previously in the *Bordetella* genus. *B. bronchiseptica* and *B. parapertussis* possess the *ptx* locus but pertussis toxin is not expressed in these species (Arico and Rappuoli, 1987; Nicosia and Rappuoli, 1987). *B. avium* does not contain sequences related to the *ptx* or *cya* genes yet is flagellate whereas *B. parapertussis* and *B. pertussis* are not, suggesting a possible evolutionary tree (Arico and Rappuoli, 1987; Brownlie *et al.*, 1988). These results thus provide a starting point for work where *Bordetella* flagellin and surrounding sequences can be compared. Loss of activity may be at the transcriptional level and examination of promoter sequences would be useful to determine if changes there have altered expression of the flagellin genes. If the mutation in the flagellin gene of *B. pertussis* and *B. parapertussis* are similar, this would indicate that loss of flagellin expression occurred before they diverged.

CHAPTER 7

DETERMINATION OF THE METHYLATION STATE OF *BORDETELLA* DNA.

7.1. Introduction.

Methylation has been shown to be involved in phase variation for example in the control of gene expression in *E. coli* pyelonephritis-associated pili (Blyn *et al.*, 1990). It would be useful to determine if methylation is involved in the expression of *Bordetella* virulence factors or even flagellin. Two commonly examined methylation states are Dam and Dcm methylation. The Dam methylase of *E. coli* methylates at the N⁶ position of adenine in the sequence 5'-GATC-3' by transferring a methyl group from S-adenosylmethionine (Hattman *et al.*, 1978). The Dcm methylase adds methyl groups to the C⁵ position of the internal cytosines in the sequences 5'-CCAGG-3' and 5'-CCTGG-3' (May and Hattman, 1975, Table 7.1).

The following restriction enzymes will not cleave DNA that has been Dam or Dcm methylated, Dam: *Mbo*I; Dcm: *Eco*RII. Isoschizomers of these particular enzymes Dam: *Sau*3A; Dcm: *Bst*NI are able to cleave DNA irrespective of Dam or Dcm methylation respectively (Table 7.1, noted by *). The enzyme *Dpn*I cleaves only Dam-methylated DNA. Several restriction endonuclease isochizomers differ in their sensitivity to methylation when other sites than Dam or Dcm are modified. Isoschizomer pairs and modified recognition sites at which they differ are shown in Table 7.1.

The methylation state of *Bordetella* phase variants was first examined by Goldman *et al.* (1987). This work suggested that phase I and X-mode *B. pertussis* strains were modified, possibly due to methylation, resulting in the resistance of their DNA to certain restriction enzymes. One enzyme *Msp*I did not digest the DNA of phase I strains whereas it did digest that of phase IV strains. However MacGregor (1990) found no digestion of the DNA of either *B. pertussis* phase variant with *Msp*I, but digestion of both with *Hpa*II, its isoschizomer. The lack of digestion with *Msp*I could be due to methylation at the site CCGG. MacGregor (1990) observed that, on *Mbo*I digestion, several fragments of approximately 1.5-3 kb were missing from the avirulent strains. In the work reported here,

Table 7.1. Isochizomers and difference in sensitivity to methylated DNA at particular modified sites.

Methylated sequence	Restriction isoschizomer pair	
	Digested by	Not digested by
G ^{m6} ATC	<i>Sau3A</i> *	<i>MboI</i> *
GAT ^{m5} C	<i>MboI</i>	<i>Sau3A</i>
GAT ^{m4} C	<i>MboI</i>	<i>Sau3A</i>
m ⁴ CCGG	<i>MspI</i>	<i>HpaII</i>
C ^{m5} CGG	<i>MspI</i>	<i>HpaII</i>
C ^{m4} CGG	<i>MspI</i>	<i>HpaII</i>
m ⁵ CCGG		<i>MspI/HpaII</i>
hm ⁵ C ^{hm5} CGG		<i>MspI/HpaII</i>
C ^{m5} CA/TGG	<i>BstNI</i> *	<i>EcoRII</i> *
hm ⁵ C ^{hm5} CA/TGG		<i>BstNI/EcoRII</i>
m ⁵ CCA/TGG	<i>BstNI/EcoRII</i>	
m ⁵ C ^{m5} CA/TGG	<i>BstNI</i>	
C ^{m4} CA/TGG		<i>EcoRII</i>
m ⁴ CCA/TGG		<i>EcoRII</i>
CC ^{m6} AGG		<i>EcoRII</i>

m⁴C = N4-methylcytosine; m⁵C = C5-methylcytosine

hm⁵C = hydroxymethylcytosine; m⁶A = N6-methyladenine

A/T = A or T base present

restriction digests with enzymes sensitive to methylated DNA were repeated and extended by examination of DNA from a number of strains of the four species of *Bordetella*.

7.2. Examination of *Bordetella* DNA for methylation.

The DNA from strains of all four *Bordetella* species was digested by the enzymes *Sau3A*, *MboI*, *DpnI*; *MspI*, *HpaII*; *BstNI* and *EcoRII* to examine the methylation state of the DNA. All strains were grown under X mode conditions, except for *B. pertussis* strain Taberman which was also grown under C mode conditions. Both *Sau3A* (Fig. 7.1, A) and *MboI* (Fig. 7.1, B) digested the *Bordetella* DNA equally well whereas the enzyme *DpnI* (Fig. 7.1, C) was not found to digest any of the *Bordetella* DNA, showing the absence of Dam methylation. No fragments were found to be missing contrary to the report of MacGregor (1990).

Bordetella DNA from all four species was fully digested by the enzymes *MspI* (Fig. 7.2, A) and *HpaII* (Fig. 7.2, B) with two exceptions. One exception was C-mode *B. pertussis* strain Taberman DNA which showed a high molecular weight single band (Fig. 7.2, lane 5) with *MspI* digestion but not *HpaII* digestion and which is not present in X-mode Taberman DNA with *MspI* digestion (Fig. 7.2, lane 4). The other exception was *B. parapertussis* 10520 (Fig. 7.2, A, lane 25) which was not completely digested with *MspI*. Both cases could be showing some methylation at CCGG sites. In contrast to the results of MacGregor (1990), the majority of the *Bordetella* DNA was digested with *MspI* and unlike the report of Goldman et al. (1987), C-mode DNA as opposed to the X-mode DNA was showing some methylation sensitivity and no differences were found between *B. pertussis* phase I or phase IV DNA. The discrepancy in the *MspI* results on *B. pertussis* DNA does not appear to be due to any variation in the growth of the original strains as they were grown under the same conditions in both studies. Known methylated sites which inhibit *MspI* digestion also inhibit *HpaII*

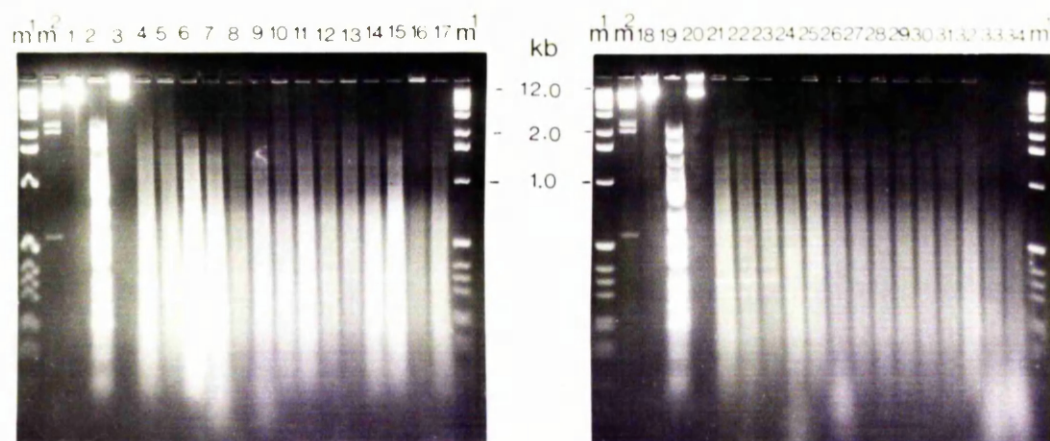
Fig. 7.1. *Sau3A*, *MboI* and *DpnI*-digested *Bordetella* chromosomal DNA.

The mol. wt. markers are noted by m^1 = kilobase marker, and m^2 = lambda *HindIII*. Lanes contained DNA prepared from the following strains and digested with (Fig. A) *Sau3A*; (Fig. B) *MboI* and (Fig. C) *DpnI* (except where stated):

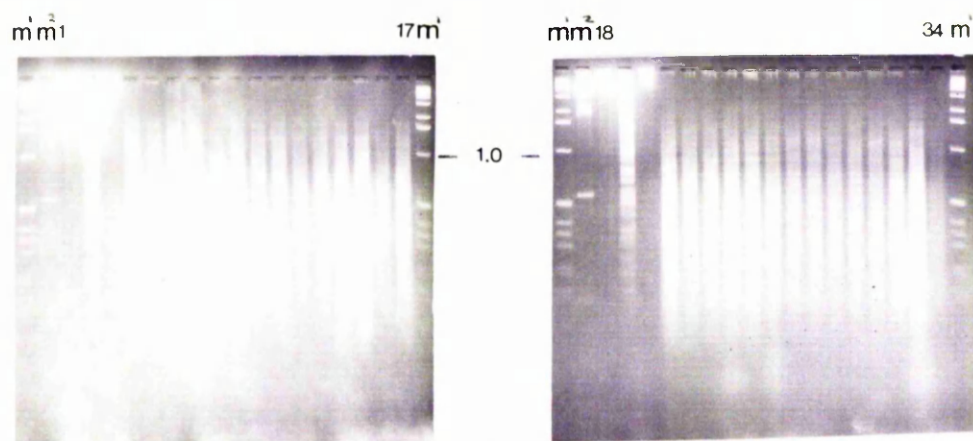
(1 and 18) lambda DNA (undigested); (2 and 19) lambda DNA;

B. pertussis: (lanes 3, 20) Taberman (undigested); (4, 21) Taberman; (5) Taberman "C-mode"; (6) Tohama; (7) Wellcome 28; (8) Arg 40; (9) Arg 41; (10) L84 I; (11) L84 IV; (12) 134 I; (13) 134 IV; (14) D 30042 I; (15) D 30042 IV; (16) 364 IV; (17) 11615 IV; (22) M2 I; (23) M2 I; *B. parapertussis*: (24) 5952; (25) 10520; *B. bronchiseptica*: (26) 452; (27) A51 I; (28) A51 III; (29) 276 I; (30) 276 III; (31) FW5 I; (32) FW5 III; *B. avium*: (33) 4148; (34) 4480.

A



B



C

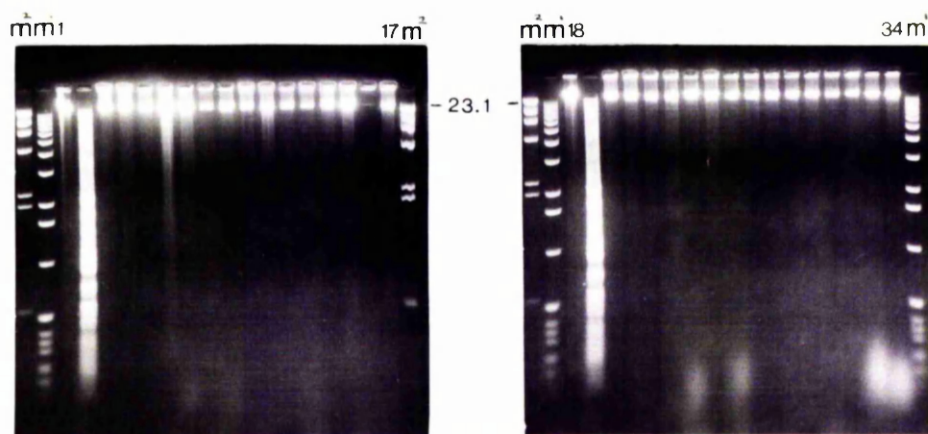
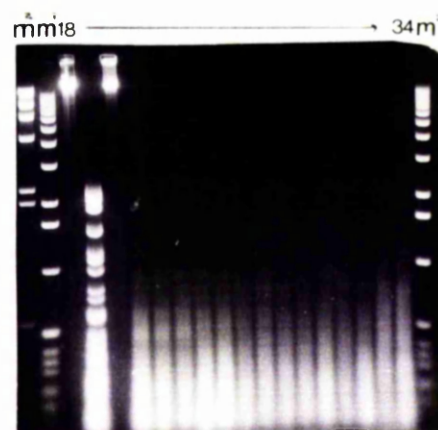
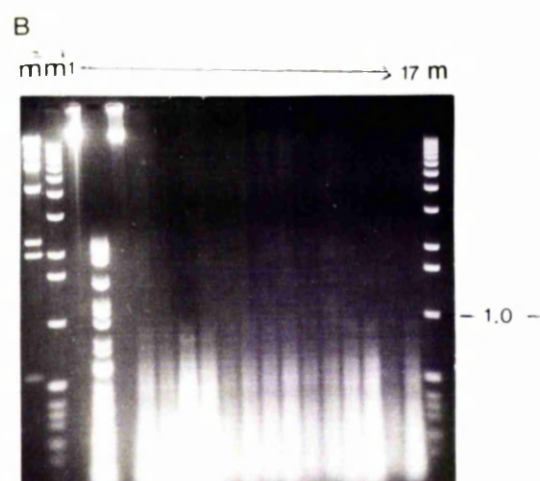
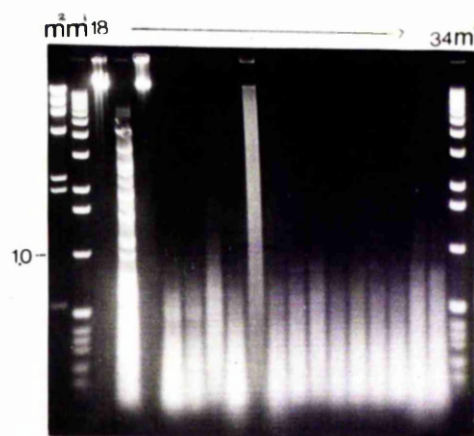
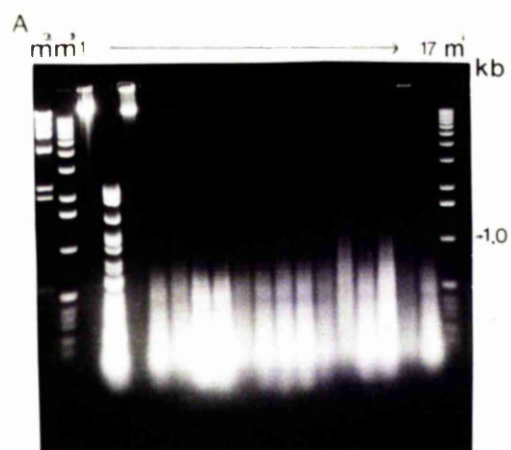


Fig. 7.2. *Msp*I and *Hpa*II-digested *Bordetella* chromosomal DNA.

The mol. wt. markers are noted by m^1 = kilobase marker, and m^2 = lambda *Hind*III. Lanes contained DNA prepared from the following strains digested with (Fig. A) *Msp*I and (Fig. B) *Hpa*II (except where indicated): (1 and 18) lambda DNA (undigested); (2 and 19) lambda DNA; *B. pertussis*: (3, 20) Taberman (undigested); (4, 21) Taberman; (5) Taberman "C-mode"; (6) Tohama; (7) Wellcome 28; (8) Arg 40; (9) Arg 41; (10) L84 I; (11) L84 IV; (12) 134 I; (13) 134 IV; (14) D 30042 I; (15) D 30042 IV; (16) 364 IV; (17) 11615 IV; (22) M2 I; (23) M2 I; *B. parapertussis*: (24) 5952; (25) 10520; *B. bronchiseptica*: (26) 452; (27) A51 I; (28) A51 III; (29) 276 I; (30) 276 III; (31) FW5 I; (32) FW5 III; *B. avium*: (33) 4148; (34) 4480.



digestion, but there was no inhibition of *HpaII* in any of the results. Thus if there is any methylation at a CCGG site, the exact position is not known.

Digestion with *EcoRII* (Fig. 7.3) and *BstNI* (Fig 7.4) showed interesting results. Most of the DNA samples were digested by both *EcoRII* and *BstNI*, but neither enzyme digested Taberman DNA when prepared after growth of the strain in C-mode conditions (Fig. 7.3 lane 5, Fig. 7.4. lane 5). The DNA of *B. parapertussis* strain 10520 was not digested to completion by either enzyme (Fig. 7.3 lane 23, Fig. 7.4 lane 24). The DNA of two of the *B. pertussis* strains 134 IV and 364 IV was not found to be digested by *EcoRII* (Fig. 7.3. lane 13 and 16) but was digested by *BstNI* (Fig. 7.4. lane 13 and 16). The DNA of *B. pertussis* strain D30042 IV was not fully digested with *BstNI* (Fig. 7.4. lane 15).

7.3. Discussion.

An absence of extensive Dam methylation was shown by *MboI* and *BscI* cleaving the *Bordetella* chromosomal DNA. If the *BscI* recognition sequence (5'-AT'CGAT-3') were preceded by a 5' guanine or followed by a 3' cytosine, the Dam transmethylase would have recognised the 5'-GATC-3' sequence and methylated the adenine to form N⁶ methyladenine. These *BscI* sites would not have been cleaved.

The C-mode *B. pertussis* Taberman DNA high molecular weight single band that is not cleaved with *MspI* but is by *HpaII* is of interest as it suggests specific CCGG sites for methylase action. The *B. pertussis* strains 134 IV and 364 IV were not digested with *EcoRII*, but were digested with *BstNI* suggesting the site C^{m5}CA/TGG (Table 7.1.) may be occurring due to Dcm methylation of the DNA. The DNA of *B. pertussis* 30042IV was completely digested by *EcoRII* but there was some inhibition of *BstNI* digestion. However, the inhibition was not complete and thus all the sites do not appear to be fully methylated. The lack of digestion of Taberman DNA (C-mode) and only partial digestion of *B. parapertussis* 10520 by *BstNI* and *EcoRII* could be due to the presence of

Fig. 7.3. *Eco*RII-digested *Bordetella* chromosomal DNA.

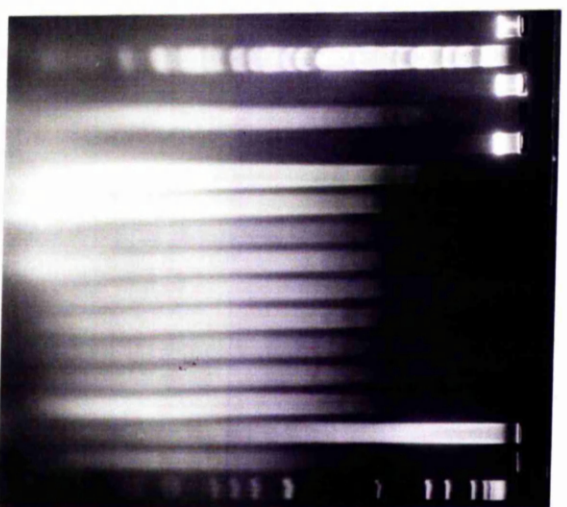
The mol. wt. (kilobase, Gibco-BRL) marker (m) sizes are indicated between the two agarose gels. Lanes contained DNA prepared from the following strains digested with *Eco*RII (except were indicated): (1 and 17) lambda DNA (undigested); (2 and 18) lambda DNA;

B. pertussis: (3) Taberman (undigested); (4) Taberman; (5) Taberman "C-mode"; (6) Tohama; (7) Wellcome 28; (8) Arg 40; (9) Arg 41; (10) L84 I; (11) L84 IV; (12) 134 I; (13) 134 IV; (14) D 30042 I; (15) D 30042 IV; (16) 364 IV; (19) 11615 IV; (20) M2 I; (21) M2 I; *B. parapertussis*: (22) 5952; (23) 10520; *B. bronchiseptica*: (24) A51 I; (25) A51 III; (26) 276 I; (27) 276 III; (28) FW5 I; (29) FW5 III; (31) 452; (30) *B. avium* 4480.

Fig. 7.4. *Bst*NI-digested *Bordetella* chromosomal DNA.

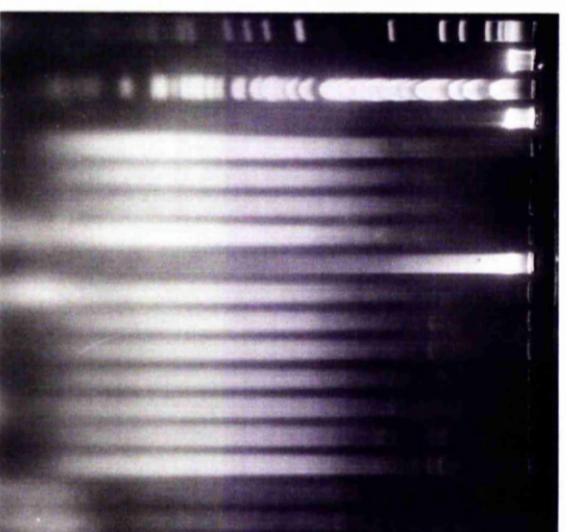
The mol. wt. (kilobase) marker is indicated between the two agarose gels. Lanes contained DNA prepared from the following strains digested with *Bst*NI (except where indicated):

(1 and 17) lambda DNA (undigested); (2 and 18) lambda DNA;
B. pertussis: (3) Taberman (undigested); (4) Taberman; (5) Taberman "C-mode";
(6) Tohama; (7) Wellcome 28; (8) Arg 40; (9) Arg 41; (10) L84 I; (11) L84 IV;
(12) 134 I; (13) 134 IV; (14) D 30042 I; (15) D 30042 IV; (16) 364 IV; (19) 11615
IV (undigested); (20) 11615 IV; (21) M2 I; (22) M2 I; *B. parapertussis*: (23) 5952;
(24) 10520; *B. bronchiseptica*: (25) 452; (26) A51 I; (27) A51 III; (28) 276 I; (29)
276 III; (30) FW5 I; (31) FW5 III; *B. avium*: (32) 4148; (33) 4480.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 m

— 2.0 —
— 1.0 —



m17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

hm⁵C^{hm5}CA/TGG sites (Table 7.1.). The DNA of *B. parapertussis* strain 10520 may not have been fully digested due to some, but not all, of the CCA/TGG sequences being methylated. Possibly the partial methylation involves a subset of CCA/TGG sequences that would correspond to methylation only at CCA/TGG sequences within a particular recognition sequence, such as GCCA/TGGC. This contrasts with the other *B. parapertussis* strain 5952 that does not show methylation. The flagellar homology results in the previous chapter showed the *B. parapertussis* strain 5952 to have a very similar hybridisation profile to *B. bronchiseptica*, but, the *B. parapertussis* strain 10520 differed in having an extra homologous fragment. This was shown after *Cla*I and *Sal*I digestion, and although this may have been due to methylation it may equally have been due to the DNA not being totally purified.

As not all the phase IV *B. pertussis* strains showed evidence of methylation, this suggests a potential for variation between the strains. McGillivray *et al.* (1989) suggested that not all phase variants necessarily arise by spontaneous frame-shift mutations. This was based on the hybridisation of *Bordetella* chromosomal DNA to a probe which contained the *bvg* locus of *B. pertussis*. Hybridisation of *Eco*RI-digested DNA from virulent and avirulent variants of both *B. pertussis* (including strain L84 I and IV) and *B. bronchiseptica* showed no detectable difference, except for one strain, 11615 IV, where there was a different hybridisation pattern. In this study the DNA of neither *B. pertussis* L84 IV nor 11615 IV was found to be methylated but the DNA of two other phase IV strains not tested by McGillivray *et al.* (1989) showed evidence of methylation. It is possible that phase IV strains arrive at their avirulent state in different ways, by spontaneous frame-shift mutations (Stibitz *et al.*, 1989), by DNA rearrangement (McGillivray *et al.*, 1989) and perhaps by other methods such as methylation. In *E. coli* spontaneous deamination of 5-methylcytosine results in thymine-guanine mismatches, which may generate mutations by C to T transitions (Coulondre *et al.*, 1978).

The results, particularly with the Taberman C-mode and phase IV variants, need to be repeated. It would also be useful to test further C-modulated strains and possibly phase IV strains grown under C-mode conditions to see if they become methylated.

CHAPTER 8

DISCUSSION.

8.1. Analysis of *B. pertussis* DNA fragments proposed as homologous to *Salmonella typhimurium* Hin invertase DNA.

S. typhimurium possesses two alternatively expressed flagellins H1 and H2. Expression is determined by a site-specific recombination event which inverts a segment of DNA that lies adjacent to the *H2* flagellin gene (Fig. 1.6.A.). The event requires the presence of an intact *hin* gene (Szekely and Simon, 1983). The cell switches between expression of the two flagellin genes at a significant frequency such that any culture of the strain, although predominately in one phase, will contain some cells in the opposite phase.

8.1.a. Characterisation of a *B. pertussis* DNA fragment previously proposed as homologous to *S. typhimurium* Hin invertase DNA.

Previous results showed the hybridisation of a *S. typhimurium* DNA probe, which included Hin invertase DNA, to a *B. pertussis* cosmid library clone, p16, and a ~0.58 kb *Cla*I fragment in *B. pertussis* chromosomal DNA (MacGregor, 1990). In the present study, it was confirmed that the p16 clone contained a 0.58 kb *Cla*I fragment, and it was also shown that when the fragment was used as a probe, it hybridised to a 0.58 kb *Cla*I fragment in *B. pertussis* chromosomal DNA. The 0.58 kb fragment was subcloned into M13, sequenced and the DNA sequence compared to the EMBL and Genbank DNA database by the DNASTAR programme, Nucscan. No homology to any invertase or related sequence was found. Translating the DNA sequence showed one large open reading frame to exist. This open reading frame was compared to other amino acid sequences in the NBRF protein database using the DNASTAR programme Proscan. No significant homology was found though it was interesting to find small regions of homology to insertion sequence transposases. The largest open reading frame consisted of 191 amino acids of which 139 showed some homology to a transposase. The highest region of homology was 29.4% identity in 34 amino acids (Fig. 3.10). The homologous regions appear to be too small to

be of significance but the function of this sequence could be assessed by subcloning the complete open reading frame in an expression vector and examining the protein product for transposase activity. The *Bordetella* DNA fragment thus had limited homology to *S. typhimurium* DNA in the *Hin* invertase DNA region, although presumably enough to enable the original hybridisation of the 680 bp *S. typhimurium* DNA probe to *Cla*I-digested *Bordetella* DNA. The reverse hybridisation i.e. with the 0.58 kb fragment of *B. pertussis* DNA as a probe to the *S. typhimurium* DNA however was not successful although the hybridisation conditions of both MacGregor (1990) and Foxall (1990) were used. When the photograph of the dot-blot analysis of the cosmid library probed with the 680 bp fragment by MacGregor (1990) was re-examined, several clones were shown to be hybridising to various degrees. Thus, the conditions used in the original work do not appear to have been stringent enough to be certain that p16 was the most specific clone. The exact hybridisation procedure used by MacGregor (1990) with the *S. typhimurium* *Hin* invertase DNA fragment probe against chromosomal DNA was therefore repeated.

The plasmid pKK1001B, which includes the *S. typhimurium hin* DNA and adjacent DNA, was digested with *Bsc*I and *Stu*I to produce the probe. The probe, a 680 bp fragment, consisted of the *hin* DNA (minus 47 bp from the N-terminus of the gene), and additional adjacent downstream sequences. This probe originally thought not to include flagellin sequences, hybridised to a ~0.58 kb fragment in *B. pertussis* chromosomal DNA confirming the result of MacGregor (1990). However, in this study the hybridisation was weak and there was much stronger hybridisation to a ~0.96 kb fragment. The absence of hybridisation to this larger fragment in MacGregor's work could have been due to differences in the production of the probe or in the conditions of the Southern transfer.

The specificity of the *S. typhimurium* *Hin* invertase DNA probe was examined. The *hin* DNA is unusual in that when the host, *S. typhimurium*, switches between flagellin types both orientations of the *hin* gene will occur. This

will be apparent in the digestion pattern of the DNA prepared from the culture. The *hin* gene is adjacent to the *H2* flagellin promoter, the inverted repeat right, and then the *H2* flagellin gene (Fig. 3.2). Only the N terminal first 114 bp of the *H2* flagellin gene were known (Silverman *et al.*, 1981) and there is a high degree of homology to the fully sequenced *H1* flagellin gene (Joys, 1985, Fig. 6.1.). It was therefore hypothesised that the *BscI* site present in the *H1* flagellin gene would also be present in the *H2* flagellin gene sequence. A recent report (Okazaki *et al.*, 1993) has shown this to be the case. Digestion of pKK1001B with *BscI* and *StuI* would thus produce two similar-sized fragments of ~680 bp which would co-migrate on electrophoresis. One (*BscI-StuI*) fragment (of 678 bp) would consist of 526 bp of the already recognised *Hin* invertase DNA region. The other would be the adjacent sequence, a *StuI-BscI* fragment, of 689 bp including the *H2* gene promoter, right inverted repeat and N-terminus of the *H2* structural gene. The probe made from the ~680 bp region would thus consist of all these components and potentially give rise to confusing hybridisation results.

On digestion of the pKK1001B plasmid with *BscI* and *StuI* a further fragment of 620 bp is produced in addition to the 680 bp. This fragment was also used in this study as a probe against chromosomal *Bordetella* DNA digested with *ClaI*. Interestingly, the 620 bp probe also hybridised to the same *Bordetella* fragments i.e. 0.96 kb and 0.58 kb as did the "680 bp" fragment probe although great care had been taken to ensure they were not cross-contaminated. The occurrence of the ~620 bp fragment would be explained by the presence of a *BscI* site in the *H2* flagellin gene and the fact that, on inversion of the *hin* gene, the *BscI* site from the *hin* DNA would then be closest to the *H2* flagellin gene resulting in a *BscI/BscI* fragment of 617 bp. Thus the ~620 bp fragment would contain 47 bp of the *hin* gene, the right inverted repeat and 479 bp of the *H2* flagellin gene (Fig. 3.2). In addition, the "680 bp" fragment consisting of two fragments of very similar size, would explain its greater intensity as compared to the 620 bp fragment on ethidium bromide-stained gels.

Analysis of the *S. typhimurium* *hin* sequence and the surrounding sequences allowed calculations of fragment sizes assuming a *BscI* site in the *H2* flagellin gene sequence at the same position as in the *H1* gene sequence. Restriction enzyme digestion would thus produce fragments of ~680 bp and ~620 bp, each containing similar DNA regions, giving the same hybridisation pattern to occur with both the 680 and 620 bp probes as was shown in this study. The 680 bp probe would consist not only of the *Hin* invertase DNA but also the *H2* promoter, inverted repeat right and 479 bp of the *H2* flagellin gene. As the 620 bp probe would not contain the *H2* promoter, the hybridisation must be due to the DNA invertase sequences, right inverted repeat and or flagellin region (N-terminus). More precisely-defined probes were produced to determine to which *S. typhimurium* DNA sequences the *Bordetella* DNA was homologous.

8.1.b. Identification of *Bordetella* DNA fragments hybridising to DNA invertase target sequences.

DNA invertases act on inverted repeat sequences. After examination of published invertase target sequences, six mixed oligonucleotides were designed varying in size from 15 bp to 26 bp. As probes, they hybridised to several fragments in *ClaI*-digested *Bordetella* DNA. Non-specific binding may have been due to the GC codon usage being over-emphasised or the number of possible combinations of oligonucleotide sequences which were present, which can result in the hybridisation temperature not being stringent enough for all the probe sequences present. Fewer chromosomal fragments hybridised to probes which emphasised the *S. typhimurium* and *E. coli* target sequences than did those emphasising *M. bovis* sequences. It has been suggested that, whereas IS-like elements probably exist as integral entities in just a few locations within the genome, the inverted repeat sequences are present as multiple copies (Alsheikly and Lofdahl, 1989). The pattern of hybridisation was not the same as that of the

680 bp fragment probe and thus the 680 bp homology was presumably not due to inverted repeat homology in the *Bordetella* DNA.

8.1.c. Characterisation of a *B. pertussis* DNA fragment hybridising to the *hin* gene probe.

The *hin* gene was amplified by PCR from *S. typhimurium* DNA in pKK1001B. It was then used as a probe against *B. pertussis* *Cla*I-digested chromosomal DNA and this resulted in hybridisation to a ~1.1 kb fragment. Although this was a similar sized fragment to the one which hybridised to the "0.68 kb" fragment, ~0.96 kb, the hybridisation to the ~1.1 kb was not as strong. Digestion of the *Bordetella* DNA with a different enzyme, *Sal*I confirmed that the probes were hybridising to different fragments as the 680 bp fragment hybridised to a 1.6 kb *Sal*I fragment whereas the PCR amplified *hin* gene probe hybridised again to a ~1.1 kb *Sal*I fragment. The hybridisation of the 680 bp fragment probe thus could not have been due to homology to an invertase or inverted repeat and may therefore have been due to homology to the flagellin sequence.

The hybridisation of *hin* gene to ~1.1 kb *Cla*I and *Sal*I fragments in *B. pertussis* DNA suggested that it had homology to the 1.05 kb tandem repeats described by Glare *et al.*, (1990). Sequence comparison of the *hin* gene and the tandem repeat showed two small regions of high homology with 75% identity over 20 bases. One of these regions, a 17 bp sequence, was used to design a probe and it was shown to hybridise to the *Bordetella* DNA. In addition, the *hin* gene probe was also shown to hybridise to the other *Bordetella* species. A *Cla*I fragment in the DNA of the *B. avium* strain, the same size as that in *B. pertussis* hybridised to the probe, but, there was no evidence of tandem repeats in the *B. avium* strains studied, either on examination of ethidium bromide stained gels, or from the results of McPheat and McNally (1987a). The probe hybridised to large *Cla*I fragments of >10 kb in DNA from *B. bronchiseptica* and *B.*

parapertussis strains. The *B. pertussis* 1.05 kb tandemly repeated sequence has been identified by Glare *et al.* (1990) as showing considerable homology to a repeat sequence transposon-like insertion element, IS481 (McLafferty *et al.*, 1988). Glare *et al.* (1990) showed homology of the 1.05 kb repeat sequence probe to a single copy sequence in *B. bronchiseptica* and although the IS481 repeats have not been found in the other *Bordetella* species (McLafferty *et al.*, 1988; McPheat and McNally, 1987a,b), single copy sequences have been shown to have homology to the closely related sequences of IS481, IS481v1 and IS481v2 in *B. parapertussis* and *B. bronchiseptica* (McPheat *et al.*, 1987a). McPheat *et al.*, 1987a showed homology to two *B. bronchiseptica* fragments, although hybridisation to the 10.1 kb *Bam*H1 fragment was faint. It is possible that the homology to the DNA from *Bordetella* strains other than *B. pertussis* shown in this study was due to the presence of single insertion sequences (ISs). Other ISs have been found in *B. parapertussis*, *B. bronchiseptica* (Zee *et al.*, 1993) and *B. avium* (Savelkoul *et al.*, 1993). The location of ISs is also intriguing, as variations of IS481 have been shown to occur close to *B. pertussis* virulence genes. One is located upstream of the adenylate cyclase locus another is located immediately adjacent to the C-terminal end of the agglutinin 2 fimbrial subunit gene (McPheat *et al.*, 1989b) and one is close to the porin gene (Li *et al.*, 1991b).

The results in this study suggested that the *hin* gene probe was not hybridising to a homologous invertase DNA sequence in *B. pertussis* but rather to an insertion sequence. Hybridisation of the *S. typhimurium hin* DNA probe had also been found to the ~0.58 kb *B. pertussis* fragment which was shown here to have low sequence homology to insertion sequence/transposon transposases. Examination of the amino acid sequence between the *hin* gene and *H2* flagellin sequence open reading frames showed other reading frames in the same translation frame. Homology of the largest of these ORFs also showed limited homology to transposases, although as with the 0.58 kb sequence the areas are small the highest homology e.g. 29.2% identity in a 48 amino acid overlap. The

hypothesis outlined above would allow for this *S. typhimurium* region to have been included in the 680 bp and 620 bp probes, giving rise to the homology shown to the 0.58 kb fragment.

Although the invertases are noted for their amino-acid sequence homology to resolvases, there is also a potential homology to transposases, as invertases perhaps have a common evolutionary origin with transposons, some of which have transposase and resolvase activity (Stern and Kamp, 1989). Thus, in this study, homology has been shown between a GC-rich region of the *hin* gene and an insertion sequence and *S. typhimurium* and *B. pertussis* having potential coding areas for transposases or at least in their previous history. The similarity does not need to be extensive, merely sufficient to enable hybridisation. Other homology between the *S. typhimurium* DNA probe and the *Bordetella* DNA appeared to have been due to flagellin sequences, as described below.

8.2. Analysis of *Bordetella* DNA for homology to flagellin gene sequences.

The *H1* flagellin gene was amplified by PCR from *S. typhimurium* chromosomal DNA and used as a probe against *B. pertussis* chromosomal DNA. This probe hybridised to the same sized *Cla*I and *Sal*I fragments of *B. pertussis* DNA as did the pKK1001B "680 bp" fragment probe. Therefore, the hybridisation noted with the 680 bp probe could possibly have been due to flagellin sequences. Although the original impetus for the flagellin work was to corroborate this hypothesis, the results have provided other useful information on homology to flagellin sequences in all species of *Bordetella*.

Two flagellin DNA probes were used: the whole *S. typhimurium* *H1* flagellin gene and the 15 bp oligonucleotide derived from the conserved N-terminal sequences of various flagellins. Both showed homology to DNA from all four species of *Bordetella* despite only two of them being motile. This indicates the presence of non-expressed flagellin genes in the two non-motile species (*B. pertussis* and *B. paraptussis*). Other workers, (Akerley *et al.*, 1992),

mentioned that *B. pertussis* chromosomal DNA contained sequences which hybridised to degenerate oligonucleotide probes based on the amino acid sequences of *B. bronchiseptica* flagellin. The work reported here and elsewhere (Leigh *et al.*, 1993) supports that observation and extended the investigation to non-motile *B. parapertussis* strains and motile *B. avium* strains by showing that they too possessed homology with the *S. typhimurium* flagellin gene sequences. These conclusions are supported by the most recent report from Akerley *et al.* (1993).

The flagellin gene, named *flaA*, from *B. bronchiseptica* and its upstream region has now been sequenced (Akerley *et al.*, 1993). The predicted flagellin protein sequence has a high degree of amino acid identity with the FliC proteins of *S. typhimurium* and *E. coli* especially at the amino and carboxy termini. The amino acid sequence similarity between the flagellins of *B. bronchiseptica* and *S. typhimurium* is unexpectedly greater than the similarity between *E. coli* and *S. typhimurium* FliC proteins, hence explaining the high level of hybridisation using the *H1* (*fliC*) flagellin DNA probe in this study. The homology of the *Bordetella* protein with those of *E. coli* and *S. typhimurium* is not unusual in that the amino-terminal sequences of the fimbrial AGG subunits have been shown to share 50% homology with that of *E. coli* K99 fimbrial subunits (Mooi *et al.*, 1987). The extensive conservation of flagellin sequences at the amino and carboxy termini has been noted for a variety of genera (Joys, 1988) and it has been suggested that these domains are essential for filament formation (Kuwajima, 1988).

The regulatory hierarchy in *E. coli* and *S. typhimurium* acts to ensure that the highly-expressed filament structural protein, flagellin, is synthesised only after a prerequisite set of other structural proteins has been expressed and properly assembled. The hybridisation work in this study and the sequence similarity with *S. typhimurium* and *E. coli* sequences suggests that additional features for the production of the flagellin may be conserved in the bordetellae.

Sequencing upstream of the flagellin gene *flaA* in *B. bronchiseptica* has detected a locus named *frl*. The *frl* locus efficiently complemented mutations in the *flhDC* loci of *E. coli*. The *flhD* and *flhC* genes of *E. coli* are early genes of the flagellar hierarchy that encode products required for transcription of middle and late genes. The *flaA* transcriptional start site in *B. bronchiseptica* maps to a consensus recognition sequence for an alternative sigma factor. This could suggest that transcription from the *flaA* promoter requires one or more products of the *frl* locus and the product of the *fliA* gene. The *fliA* gene in *S. typhimurium* encodes the alternative sigma factor. It is a middle gene which is required for transcription of late gene promoters, including the promoter for flagellin (*H1*, *fliC*). The transcription of early genes is important in *S. typhimurium*, since if an early gene did not function it would result in late genes not being expressed and so there would be no filament formation. Analysis of *flaA* mRNA production by Northern (RNA) blotting and primer extension indicated that negative regulation of flagellin expression in *B. bronchiseptica* by BvgAS occurs at the level of transcription. Thus the *Bordetella* virulence control system would appear to mediate transcriptional control of *flaA* through a regulatory hierarchy that includes the *frl* locus and an alternative sigma factor (Akerley *et al.*, 1993). Many bacteria appear to utilise an alternative sigma factor to direct transcription of flagellin and expression of motility-associated loci (Arnosti and Chamberlain, 1989). Interestingly, as a further control in *E. coli*, there is an anti-sigma factor *flgM* which binds to the alternative sigma factor and disturbs its ability to form a complex with RNA polymerase core enzyme. This novel type of negative regulator could possibly be present in *Bordetella* species.

The functional role of motility during respiratory infection by *B. bronchiseptica* requires examination. *B. bronchiseptica* growing at 25°C would have low concentrations of BvgA present and the vir-repressed genes (*vrg*) including flagellin expression would be active. On transmission to a susceptible host the flagella could provide a means of moving through the mucosal blanket

in the nose and trachea. As the temperature surrounding the *Bordetella* increased, active BvgS would increase, activating BvgA the transcriptional activator, and turning off the *vrg* genes including the flagellin genes. The *vir*-activated genes would now be expressed allowing attachment and production of toxins. The flagellin genes would not be needed again until the bacteria encountered a new host. Although *B. pertussis* does not produce flagella, the production of flagella by *B. avium* and *B. bronchiseptica* in the avirulent or C-mode state could be important in a pathologically-dormant form associated with the later stages of infection and in carriers. In this situation they could serve as a reservoir of bacteria until the phase switched back to the virulent stage and the bacteria become able to initiate new infections in susceptible hosts (Weiss and Falkow, 1984).

8.3. Examination of the methylation state of *Bordetella* DNA.

The final results chapter deals with the analysis of the methylation state of the *Bordetella* DNA to determine if methylation does occur as reported by Goldman *et al.* (1987) and if there was any variance between phase (Leslie and Gardner, 1931) or antigenic mode (Lacey, 1960).

No Dam methylation was found in the *Bordetella* DNA. Results obtained with *B. pertussis* strains 134 IV and 364 IV showed that some Dcm methylation may be occurring, possibly at the site C^{m5}CA/TGG. For *B. pertussis* D30042IV, not all the sites appeared to be fully methylated. In DNA from *B. pertussis* Taberman (C-mode) and *B. paraptussis* 10520 the absence of digestion could have been due to ^{hm5}C^{hm5}CA/TGG sites. DNA from the *B. paraptussis* strain 10520 may not have been digested to completion due to some, but not all, of the CCA/TGG sequences being methylated. In contrast, DNA from the other *B. paraptussis* strain, 5952, did not show methylation.

The results, particularly with the Taberman C-mode and phase IV variants, need to be repeated. It would also be useful to test further C-modulated

strains and possibly phase IV strains grown under C-mode conditions to see if they become methylated. Dcm methylation was found only in bacteria closely related to *E. coli* e.g. *Salmonella* by Gomez-Eichelmann *et al.* (1991). Since sequence homology has been found between these strains and *Bordetella* it is possible that the Dcm methylation is also present in *Bordetella*, as is suggested in this preliminary study.

Not all phase IV *B. pertussis* strains showed evidence of methylation which suggests a potential for variation between the strains. It is probable that phase IV strains arrive at their avirulent state in different ways, such as by spontaneous frame-shift mutations (Stibitz *et al.*, 1989) or by DNA rearrangement (McGillivray *et al.*, 1989) and also perhaps by mechanisms involving methylation. Dcm methylation can be mutagenic: in *E. coli*, spontaneous deamination of 5-methylcytosine results in thymine-guanine mismatches, which may generate mutations by C to T transitions (Coulondre *et al.*, 1978). Methylation may also be important as it can modulate the activity of prokaryotic transposable elements (Roberts *et al.*, 1985).

8.4. Conclusions.

Two potential problems about hybridisation that this work has highlighted are firstly the dangers of using ill-defined probes to seek homologous gene sequences in other species. Secondly the fact that hybridisation can be obtained with only a small portion of a probe which may result in positive hybridisation to sequences that, overall, do not bear close homology to the probe. These problems were resolved by the use of PCR-amplified gene probes enabling hybridisations to be more specific aiding easier interpretation of the results. It was hypothesised that the sequences which hybridised though having small sequence homology could have some evolutionary significance.

In this study, evidence has been given to show homology between an invertase and the *B. pertussis* IS repeat sequence. The exact function of insertion

sequences (ISs) are unknown although they appear to have been important in evolution by mediating chromosomal rearrangements such as deletions, inversions and duplications and by altering the expression of adjacent genes (Iida *et al.*, 1983). There is a high degree of conservation of *B. pertussis* fragments bearing the repetitive sequence which implies that the repeat (IS481) is not involved in DNA rearrangements (Glare *et al.*, 1990). The loss of expression of adenylate cyclase and agglutinin 2 in *B. pertussis* strains was shown not to be the result of DNA rearrangements at the sites of IS481v1 or IS481v2 (McPheat *et al.*, 1989a). However, chromosomal DNA rearrangement at another uncharacterized locus between phase variants of a *B. pertussis* strain had been observed (McPheat and McNally, 1987b). This may indicate that different members of the IS family differ with regard to their ability to cause DNA rearrangements.

The presence of the *Bordetella* IS may act as markers for the original primordial acquisition of virulence factors, including the flagellin genes, through movement of the transposable elements. Insertion sequences have been shown 3 kb upstream of the adenylate cyclase gene and adjacent to the 3' end of the gene for Agglutinin 2 (McPheat *et al.*, 1989b), although their close association to flagellin sequences has not yet been shown. The origin of the *Bordetella* flagellin genes and invertase-like or insertion sequences may be examined in the light of suggestions regarding the evolution of *S. typhimurium* flagellar phase variation (Section 1.9.1.a, Silverman and Simon, 1983). The fact that there is homology between various flagellar genes suggests that these particular functions were so essential to primordial bacterial growth that they were established early in evolution (Arnosti and Chamberlain, 1989). The structure of the invertible region of *S. typhimurium* is reminiscent of the structure of IS elements in that it is flanked by 14 bp inverted repeats. In this study, hybridisation homology was shown between *Bordetella* DNA and the *S. typhimurium* *hin* gene, the *H1* flagellin gene and possibly the region between them (Section 8.1). In addition,

McPheat *et al.* (1988) described a 40 bp sequence internal to the IS in *B. pertussis* which shows some homology to the recognition sequence, the inverted repeat, of the *Hin* invertase DNA. This could also be an additional explanation of why the inverted repeat sequence oligonucleotide probes gave so many fragments of homology, due to hybridisation to the repeats in the *B. pertussis* genome.

Non-expressed flagellin genes have been shown, in this study, to almost certainly occur in *B. pertussis* and *B. parapertussis* strains. The presence of non-expressed genes has been shown previously in the *Bordetella* genus. *B. bronchiseptica* and *B. parapertussis* possess the *ptx* locus but these genes are not expressed in these species due to the accumulation of deleterious base changes, mainly in the promoter region, of the *ptx* operon. *B. avium* does not contain sequences related to the *ptx* genes (Arico and Rappuoli, 1987; Nicosia and Rappuoli, 1987). The presence or lack of genes and their expression has enabled suggestions as to the ancestry of the strains. The results reported here indicate that the ancestral *Bordetella* strain was flagellate without *ptx* or *cya* genes. *B. avium* having neither *ptx* or *cya* genes, is considered to be the most distantly related member of the genus. After acquisition of the *ptx* and *cya* genes, further derivatives arose to become *B. bronchiseptica* and, with a loss in mobility, *B. parapertussis* and *B. pertussis* were derived. *B. bronchiseptica* and *B. parapertussis* having lost the ability to express the *ptx* genes. These processes were presumably determined by niche-specialisation where environmental selection pressure dictated the retention or loss of motility and *ptx* gene expression. Thus, although all four *Bordetella* are well adapted respiratory tract pathogens, they have presumably evolved alternative strategies for survival and persistence both in the environment and in different vertebrate hosts.

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